II. REMARKS

A. Introduction

Applicants submit this Response in a bona fide attempt to (i) advance the prosecution of this case, (ii) answer each and every ground of objection and rejection as set forth by the Examiner, (iii) place the claims in a condition for allowance, and (iv) place the case in better condition for consideration on appeal.

As indicated above, Claims 1-7 have been amended, Claims 18-29 added and Claims 8-17 have been withdrawn.

Applicants respectfully submit that the noted amendments merely make explicit that which was (and is) disclosed or implicit in the original disclosure. The amendments thus add nothing that would not be reasonably apparent to a person of ordinary skill in the art to which the invention pertains.

B. Oath

As required by the Examiner, Applicants hereby submit a corrected declaration under 37 C.F.R. 1.67(a) identifying this application by serial number and filing date, wherein the corrected declaration claims priority from PCT/AU02/01226 rather than indicating that the present application is a continuation of that foreign application.

C. Response to Rejections

1. 35 U.S.C. § 112, second paragraph

The Examiner has rejected Claims 1-7, under 35 U.S.C. § 112, second paragraph, "because the method steps fail to achieve the goal set forth in the preamble." This rejection is primarily directed at Claims 1 and 2, as Claims 1 and 2 were independent and Claims 3-7 depended from Claim 2. To address these rejections, Applicants have amended the claims to clarify the relationship between the preambles and the recited steps. As amended, Claim 1 is independent and Claims 2-7 depend from Claim 1.

Claim 1 is directed to a method of inhibiting proteolytic conversion of inactive TGF-β to active TGF-β by the CIM6P receptor expressed on a cytotrophoblast cell. The goal of this claim

is achieved by administering a selected differentiation factor to the CIM6P receptor to inhibit proteolytic conversion of inactive TGF-β to active TGF-β by the receptor.

Applicants have also added new Claims 18-29. Claims 18-23 also depend from Claim 1, so the points raised above with regard to Claim 1 apply. With respect to the new, independent Claims 24-29, Applicants respectfully submit that the method steps claimed achieve the goals of the preambles for the reasons below.

New Claim 24 is directed to a method of improving placental growth, placental development or placental differentiation in a female mammal. New Claim 25 is directed to a method of promoting implantation of an embryo in a female mammal. New Claim 26 is directed to a method of preventing and/or treating a number of conditions associated with poor implantation, poor placental growth, poor differentiation or poor development in a female mammal. The goals of these claims are achieved by administering to the female mammal an effective amount of a selected differentiation factor.

New Claim 27 is directed to a method of promoting placental growth, development or differentiation in a female mammal. New Claim 28 is directed to a method of promoting implantation of an embryo in a female mammal. New Claim 29 is directed to a method of preventing and/or treating a number of conditions associated with poor implantation, poor placental growth, poor development or poor differentiation in a female mammal. The goals of these claims are achieved by administering to an embryo produced by in vitro fertilization an effective amount of a selected differentiation factor.

The correlation between the goals of the preambles and the claimed method steps is further supported by the demonstration that exogenous IGF-II administered to guinea pigs results in an increase in placental structural and functional capacity (see Sferruzzi-Perri et al. (2006) *Endocrinology*, 2006 Mar 23, copy enclosed). As shown in this reference, administration of IGF-II alone increases placental labyrinth cross-section and volume (the labyrinth is the placental exchange region), increases trophoblast volume and increases maternal blood spaces. One having ordinary skill in the art is aware that the guinea pig placenta is similar to the human placenta (see Roberts et al. (2001) *Placenta* **22**:177-185, particularly p. 178, first column, second paragraph, copy enclosed). Thus, administration of IGF-II under the conditions disclosed in the specification improves placental growth, development and differentiation in a female mammal known to be similar to humans.

For these reasons, it is respectfully submitted that the claims as amended and the new claims are clear and that they achieve the goals set forth in the preambles of the claims. Accordingly, Applicants request that the Examiner withdraw the § 112, second paragraph, rejection of the claims.

2. 35 U.S.C. § 112, first paragraph

The Examiner also rejected Claims 1-7, under 35 U.S.C. § 112, first paragraph, stating that they fail to comply with the enablement requirement. This rejection is primarily directed at Claims 1 and 2, as Claims 1 and 2 were independent and Claims 3-7 depended from Claim 2. Applicants have amended the claims so that Claim 1 is independent and Claims 2-7 depend from Claim 1.

As amended, Claim 1 is directed to a method of inhibiting proteolytic conversion of inactive TGF-β to active TGF-β by the CIM6P receptor expressed on a cytotrophoblast cell by administering to the cell a differentiation factor selected from the group consisting of IGF-II, a precursor of IGF-II, an isomer of IGF-II and an IGF-II analog to promote binding of the differentiation factor to the receptor and thereby inhibit proteolytic conversion of inactive TGF-β to active TGF-β by the CIM6P receptor.

Applicants respectfully submit that the specification, as filed, would enable one of ordinary skill in the art to use the invention, as claimed in Claim 1 and its dependent Claims. In particular, Example 1 of the specification provides a description of the use of exogenous IGF-II to compete with latent TGF-β in vitro for the same binding site on placental human CIM6P receptors. Similarly, Example 2 describes the use of exogenous IGF-II to inhibit activation of latent TGF-β in vitro by human TFI cells, which are cells that express the essential components of the CIM6P receptor system for converting inactive TGF-β to active TGF-β. Finally, Example 4 describes the use of IGF-II administered to pregnant mice to increase placental weight, to increase fetal weight, to increase placental thickness and to increase the ratio of fetal weight to placental weight. The data demonstrates that administration of IGF-II alone is able to have these biological effects.

Accordingly, these disclosures provided by the specification contain sufficient information to enable one of ordinary skill skilled in the pertinent art to make and use the

invention as claimed in Claim 1 and its dependent claims, and by extension, in new Claims 24-29.

The Examiner contends that undue experimentation would be necessary to determine the culture conditions appropriate for a number of mammals. Applicants respectfully disagree with this assertion. The specification discloses the conditions required in vitro and in vivo to effect the inhibition of conversion of inactive TGF-β to active TGF-β by IGF-II.

With regard to in vitro uses, Example 2 as discussed above demonstrates the administration of IFG-II to human cells. Selection of in vitro conditions for other mammals would not require undue experimentation, given that the person of ordinary skill in the art typically engages in such activities and such conditions are known in the art.

Similarly, the specification discloses the in vivo administration of IGF-II alone to pregnant mice to affect desired biological outcomes. For example, Example 4 provides directions regarding the administration of suitable concentrations of IGF-II to enhance placental growth and/or development. Therefore, Applicants respectfully assert that one of ordinary skill in the art would be readily able to select the appropriate administration conditions for other mammals, given that such conditions are known in the art.

Further, given that the specification discloses the use of IGF-II to inhibit conversion of inactive TGF-β to active TGF-β, one of ordinary skill in the art would also be able to select the appropriate conditions for the use of precursors, isomers and analogs of IGF-II, as such conditions are well known in the art.

The Examiner also stated that no matter, however, compelling the data provided is, it only suggests that treatment of IGF-II increases placental and birth weight, and that this may not be necessarily due to regulation of cytotrophoblast differentiation and migration, or promotion of implantation.

Applicants respectfully submit that these considerations are not applicable to considerations of enablement. As defined by MPEP Section 2164.01, the appropriate test is whether the disclosure provided by the specification contains sufficient information to enable one skilled in the pertinent art to make and use the invention as claimed.

Nevertheless, even though not relevant to the consideration of enablement, one having ordinary skill in the art would generally recognize the importance of trophoblast development in placentation. As an example, Applicants cite the section entitled "Trophoblast Development" in

Gude et al. (2004) *Thrombosis Research* **114**: 397-407, a review article co-authored by one of the present inventors.

Further, one of the inventors has demonstrated that exogenous IGF-II administered to guinea pigs results in an increase in placental structural and functional capacity (see Sferruzzi-Perri et al., referenced above). As reported in this citation, administration of IGF-II alone increases placental labyrinth cross-section and volume (the labyrinth is the placental exchange region), increases trophoblast volume and increases maternal blood spaces. The significance of these results is enhanced given that one having ordinary skill in the art recognizes that the guinea pig placenta is similar to that of the human (see Roberts et al., referenced above). Thus, the administration of IGF-II as described in the specification has been shown to improve placental growth, development, differentiation and differentiation in a mammal having a placenta known to be similar to human.

Finally, the Examiner cited Behr and Wang (*Eur. J. Obstet. Gynccol. Reprod. Biol.* (2004) **115 Suppl 1**:S72-6) for the proposition that not all manipulations in embryo culture are benign and that caution is warranted. Applicants respectfully submit that the issue is enablement and whether there is an undue burden of experimentation placed upon one of ordinary skill in the art to practice the invention. As discussed above, one having ordinary skill in the art would be readily able to select the appropriate culture conditions to use the invention. One having ordinary skill in the art would also recognize that the comments provided in Behr and Wang relate to the complex process of in vitro fertilization outcome, which is dependent upon numerous factors, including embryo quality, age of oocytes, age of mother, and embryonic pre-implantation development, which are not directly related to the invention as claimed.

Applicants also respectfully submit that new Claims 24-29 are likewise enabled by the specification as filed, for the reasons given below.

New Claim 24 is directed to a method of improving placental growth, placental development or placental differentiation in a female mammal. New Claim 25 is directed to a method of promoting implantation of an embryo in a female mammal. New Claim 26 is directed to a method of preventing and/or treating a number of conditions associated with poor implantation, poor placental growth, poor differentiation or poor development in a female mammal.

As described above, Example 4 of the application provides a description of the use of IGF-II administered to pregnant mice to increase placental weight, to increase fetal weight, to increase placental thickness and to increase the ratio of fetal weight to placental weight. Moreover, the specification also provides a description (see, e.g., paragraph 74) of various delivery systems for treatment with IGF-II and Example 4 provides directions regarding suitable concentrations of IGF-II that may be administered to enhance placental growth and/or development.

Further, it has been demonstrated that exogenous IGF-II administered to guinea pigs results in an increase in placental structural and functional capacity (see Sferruzzi-Perri et al., referenced above). As reported in this reference, administration of IGF-II alone increases placental labyrinth cross-section and volume (the labyrinth is the placental exchange region), increases trophoblast volume and increases maternal blood spaces. The significance of these results is enhanced given that one having ordinary skill in the art recognizes that the guinea pig placenta is similar to that of the human (see Roberts et al., referenced above). Thus, the administration of IGF-II as described in the specification has been shown to improve placental growth, development, differentiation and differentiation in a mammal having a placenta known to be similar to human

Accordingly, Applicants respectfully submit that the claimed invention can be accomplished in one embodiment by administration of IGF-II alone, and therefore an undue burden of experimentation is not required to practice the invention recited in Claims 24-26.

New Claim 27 is directed to a method of promoting placental growth, development or differentiation in a female mammal. New Claim 28 is directed to a method of promoting implantation of an embryo in a female mammal. Finally, new Claim 29 is directed to a method of preventing and/or treating a number of conditions associated with poor implantation, poor placental growth, poor development or poor differentiation in a female mammal.

Applicants also respectfully submit that the specification as filed would enable one having ordinary skill in the art to use the invention as claimed in Claims 27-29. Specifically, the specification clearly discloses the treatment of embryos to increase the success of implantation and to increase the success of formation of a viable placenta (see, e.g., paragraphs 24 and 25). In addition, Example 2 provides a description of the use of exogenous IGF-II to inhibit activation of latent TGF-β in vitro by human TFI cells. This example provides a description of the culture and

treatment conditions for in vitro cells suitable to inhibit activation of TGF-β. Further, Example 3 provides a description of suitable conditions for culturing human placental villous tissue using culture medium.

Accordingly, Applicants respectfully submit that the disclosure provided in the specification contains sufficient information to enable one having ordinary skill in the art to treat embryos as recited in new Claims 27-29.

Finally, the Examiner contends that a large amount of experimentation would be necessary to determine the culture conditions appropriate for a number of mammals. Applicants respectfully submit that the specification provides clear disclosure directed to two mammals, namely human and mouse. Further, Applicants contend that one having ordinary skill in the art often adapts conditions from one species to another and, therefore, that undue experimentation would not be required to use the invention with other mammalian species. Indeed, Applicants respectfully submit that the appropriate conditions for culturing embryos of different mammalian species are well known in the art.

One of ordinary skill in the art would also be able to practice the invention with regard to precursors, isomers and analogs of IGF-II. As discussed above, the invention as claimed can be accomplished in one embodiment by the administration of IGF-II alone. Accordingly, one having ordinary skill in the art would be able to use the invention with the claimed precursors, isomers and analogs of IGF-II, as the molecules and their use are well known in the art.

3. 35 U.S.C. § 102(b)

The Examiner rejected Claims 1-6 under 35 U.S.C. § 102(b) in view of the O'Neil reference. The Examiner asserts that O'Neil teaches the administration of human recombinant IGF-II to mice embryos and discloses the effect of that treatment on the proportion of two-cell stage embryos that develop to the blastocyst stage. Applicants respectfully request that the Examiner reconsider and withdraw the rejection.

As discussed above, Applicants have amended Claim 1, the primary independent claim, so that it requires inhibiting the proteolytic conversion of inactive TGF-β by administering a sufficient amount of the claimed differentiation factor to bind the CIM6P receptor.

In contrast, the O'Neil reference has no teaching regarding the use of IGF-II to inhibit proteolytic conversion of inactive TGF-β to active TGF-β by the CIM6P receptor expressed on a

cytotrophoblast cell. As such, Applicants respectfully submit that this reference fails to disclose the invention as claimed and request that the Examiner withdraw the rejection of Claims 1-6 in view of O'Neil.

With regard to the new independent claims, Claims 24-26 are directed to the administration of the claimed differentiation factors to a female mammal. However, O'Neil discloses only the administration of compounds to an embryo. Accordingly, Applicants respectfully submit that Claims 24-26 are not anticipated by O'Neil.

As discussed above, Claims 27-29 are directed to the administration of the claimed differentiation factors to an embryo. New Claim 27 is directed to a method of promoting placental growth, development or differentiation in a female mammal. New Claim 28 is directed to a method of promoting implantation of an embryo in a female mammal. Finally, new Claim 29 is directed to a method of preventing and/or treating a number of conditions associated with poor implantation, poor placental growth, poor development or poor differentiation in a female mammal.

Since O'Neil discloses the treatment of embryos from mice with human recombinant IGF-II to affect the proportion of two-cell stage embryos that develop to the blastocyst stage, the reference fails to disclose a method for treating embryos with a differentiation factor to effect placental development, to promote implantation of an embryo, or to prevent and/or treat a disease associated with altered placental growth, development or differentiation. Accordingly, Applicants respectfully submit that Claims 27-29 are not anticipated by O'Neil.

Next, the Examiner rejected Claims 1, 2, 4, 5 and 7 under 35 U.S.C. § 102(b) in view of Gluckman et al. The Examiner cites this reference for teaching the administration of IGF-II to a pregnant female. Gluckman et al. specifically discloses the administration of IGF-I or IGF-II to pregnant female rats to improve fetal weight. The reference discloses that treatment with IGF-I results in a reduction in the negative correlation between mean fetal weight and litter size. Applicants respectfully request that the Examiner reconsider and withdraw these rejections.

As discussed above, Applicants have amended Claim 1, the primary independent claim, so that it requires inhibiting the formation of active TGF-β by administering an amount of the claimed differentiation factor sufficient to bind the CIM6P receptor.

However, the Gluckman et al. reference has no teaching regarding TGF-β and the proteolytic conversion of TGF-β by the CIM6P receptor. Accordingly, there is no disclosure

related to the administration of the claimed differentiation factors to bind the CIM6P receptor and inhibit the formation of active TGF-β.

Gluckman et al. is primarily directed to the use of IGF-I, and merely hypothesizes that IGF-II would act in a similar manner. See, Gluckman et al., col. 3, lines 46-51. However, as disclosed in the specification at paragraph 82, for example, IGF-I exhibits very poor affinity for the CIM6P. As such, IGF-I would not be a suitable compound for competitively excluding latent TGF-β. Therefore, the Gluckman et al. reference's discussion regarding the use of IGF-I and IGF-II as similar compounds having similar effects does not constitute a disclosure of the use of IGF-II or the other claimed differentiation factors to interact with the CIM6P receptor and inhibit the proteolytic conversion of TGF-β. As such, Applicants respectfully submit that Claim 1 and all of its dependents are not anticipated by the Gluckman et al. reference.

With regard to the new independent claims, Claims 24-26 are directed to the administration of the claimed differentiation factors to a female mammal. New Claim 24 is directed to a method of improving placental growth, placental development or placental differentiation in a female mammal. New Claim 25 is directed to a method of promoting implantation of an embryo in a female mammal. New Claim 26 is directed to a method of preventing and/or treating a number of conditions associated with poor implantation, poor placental growth, poor differentiation or poor development in a female mammal.

However, O'Neil discloses the aclnlinistration of IGF-I or IGF-II to pregnant female rats to improve fetal weight. The document discloses that treatment with IGF-I results in a reduction in the negative correlation between mean fetal weight and litter size and reports that IGF-I does not appear to increase placental size. Accordingly, Applicants respectfully assert that Gluckman et al. does not disclose the use of the claimed differentiation factors to promote placental growth, development or differentiation, to promote implantation or to prevent and/or treat implantation failure, recurrent spontaneous miscarriage, pre-eclampsia, and placental abruption as claimed. Indeed, Gluckman teaches away from the current invention by disclosing that IGF-I does not appear to increase placental size. For these reasons, Claims 24-26 are not anticipated by Gluckman et al.

As discussed above, Claims 27-29 are directed to the administration of the claimed differentiation factors to an embryo. Since Gluckman et al. only discloses administration to a

female rat, Applicants respectfully submit that this reference fails to disclose treatment of embryos and that Claims 27 to 29 are not anticipated by Gluckman et al.

4. 35 U.S.C. § 103(a)

Finally, the Examiner rejected Claim 7 under 35 U.S.C. § 103(a) as being unpatentable in view of the O'Neil reference. The Examiner recognizes that O'Neil fails to disclose the administration of IGF-II to the specific mammalian embryos cited in Claim 7. However, the Examiner asserts that it would have been obvious to extend the applicability of its teaching from mouse embryos to other mammalian embryos. Applicants respectfully request that the Examiner reconsider and withdraw this rejection.

As discussed above, Claim 1, the primary independent claim has been amended to require inhibiting the proteolytic conversion of inactive TGF-β by administering an amount of the claimed differentiation factor sufficient to bind the CIM6P receptor. By extension, Claim 7 which depends from Claim 1 has the same requirements. Since the O'Neil reference has no teaching regarding the use of IGF-II to inhibit proteolytic conversion of inactive TGF-β to active TGF-β by the CIM6P receptor expressed on a cytotrophoblast cell, it fails to suggest the invention of Claim 1 and any of its dependents, including Claim 7. Accordingly, Applicants respectfully request that the Examiner withdraw the rejection of Claim 7 in view of O'Neil.

With regard to the new independent claims, Claims 24-26 are directed to the administration of the claimed differentiation factors to a female mammal. As described above, O'Neil discloses only the administration of compounds to an embryo. Accordingly, Claims 24-26 are not suggested by O'Neil.

Further, Claims 27-29 are directed to the administration of the claimed differentiation factors to an embryo. New Claim 27 is directed to a method of promoting placental growth, development or differentiation in a female mammal. New Claim 28 is directed to a method of promoting implantation of an embryo in a female mammal. Finally, new Claim 29 is directed to a method of preventing and/or treating a number of conditions associated with poor implantation, poor placental growth, poor development or poor differentiation in a female mammal.

Since O'Neil discloses the treatment of embryos from mice with human recombinant IGF-II to affect the proportion of two-cell stage embryos that develop to the blastocyst stage, the reference fails to suggest a method for treating embryos with a differentiation factor to effect

placental development, to promote implantation of an embryo, or to prevent and/or treat a disease associated with altered placental growth, development or differentiation. Accordingly, Applicants respectfully submit that Claims 27-29 are not obvious in view of the O'Neil reference.

III. CONCLUSION

Applicants, having answered each and every ground of rejection as set forth by the Examiner, and having added no new matter, believe that this response clearly overcomes the references of record and renders the claims clear and definite, and now submit that all claims in the above-referenced patent application are in condition for allowance and the same is respectfully solicited.

If the Examiner has any further questions or comments, Applicants invite the Examiner to contact their Attorneys of record at the telephone number below to expedite prosecution of the application.

Respectfully submitted,
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Growth and function of the normal human placenta

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Abstract The placenta is the highly specialised organ of pregnancy that supports the normal growth and development of the fetus. Growth and function of the placenta are precisely regulated and coordinated to ensure the exchange of nutrients and waste products between the maternal and fetal circulatory systems operates at maximal efficiency. The main functional units of the placenta are the chorionic villi within which fetal blood is separated by only three or four cell layers (placental membrane) from maternal blood in the surrounding intervillous space. After implantation, trophoblast cells proliferate and differentiate along two pathways described as villous and extravillous. Non-migratory, villous cytotrophoblast cells fuse to form the multinucleated syncytiotrophoblast, which forms the outer epithelial layer of the chorionic villi. It is at the terminal branches of the chorionic villi that the majority of fetal/maternal exchange occurs. Extravillous trophoblast cells migrate into the decidua and remodel uterine arteries. This facilitates blood flow to the placenta via dilated, compliant vessels, unresponsive to maternal vasomotor control. The placenta acts to provide oxygen and nutrients to the fetus, whilst removing carbon dioxide and other waste products. It metabolises a number of substances and can release metabolic products into maternal and/or fetal circulations. The placenta can help to protect the fetus against certain xenobiotic molecules, infections and maternal diseases. In addition, it releases hormones into both the maternal and fetal circulations to affect pregnancy, metabolism, fetal growth, parturition and other functions. Many placental functional changes occur that accommodate the increasing metabolic demands of the developing fetus throughout gestation.
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Introduction

The placenta is the highly specialised organ of pregnancy that, along with the fetal membranes and amniotic fluid, support the normal growth and development of the fetus. Changes in placental development and function have dramatic effects on the fetus and its ability to cope with the intrauterine environment. Implantation and the formation of the placenta is a highly coordinated process involving interaction between maternal and embryonic cells. Trophoblast cell invasion of uterine tissues and remodelling of uterine spiral arterial walls ensures that the developing feto-placental unit receives the necessary supply of blood and that efficient transfer of nutrients and gases and the removal of wastes can take place. Different types of placentation are categorised according to the number and types of layers between the maternal and fetal circulations [1]. The human placenta is a haemochorial villous organ, whereby maternal blood comes into direct contact with placental trophoblast cells and allows an intimate relationship between the developing embryo and its supply of nutrients.

This review describes the basic structural arrangements of the mature human placenta and fetal membranes and gives an overview of the processes of implantation, decidualisation and placentation that result in their formation. Finally, the main functions of the placenta are discussed under the headings of transport and metabolism, protection and endocrine function.

The structure of the mature placenta

The utero-placental unit is composed of both fetal tissue derived from the chorionic sac and maternal tissue derived from the endometrium. In the mature placenta, the fetal aspect is called the chorionic plate. This region carries the fetal chorionic blood vessels, which are branching radials from the umbilical vessels. The maternal aspect of the placenta is called the basal plate. In between these two regions is the intervillous space, which contains the main functional units of the placenta, extensively branched and closely packed villous structures containing fetal blood vessels. It is at the terminal regions of these chorionic villi that the large majority of maternal–fetal exchange occurs [2]. The intervillous space is completely lined with a multinucleated syncytium called the syncytiotrophoblast. Circulating maternal blood enters this space via spiral endometrial arteries, bathes the villi and drains back through endometrial veins. Oxygen-deficient fetal blood passes via two umbilical arteries and the branched chorionic arteries to the extensive arterio–capillary–venous system within the chorionic villi (Fig. 1A). The well-oxygenated fetal blood in the capillaries returns to the fetus via the various chorionic veins and the single umbilical vein [3].

The placental membrane

The term placental membrane (sometimes called the placental barrier) refers to the layers of cells that separate the maternal blood in the intervillous space and the fetal blood in the vasculature in the core of the villi [4]. Initially, the placental membrane is made up of four layers, the maternal facing syncytiotrophoblast, a layer of cytotrophoblast cells, connective tissue of the villus and the endothelium lining the fetal capillaries (Fig. 1B). By approximately 20 weeks, however, the cytotrophoblast cell layer of many villi becomes attenuated and disappears. Subsequently, in most of the chorionic villi, the membrane consists of three layers and, in some areas, becomes extremely thin such that the syncytiotrophoblast comes in direct contact with the fetal capillary endothelium (Fig. 1C). Thus, in these positions, the maternal blood and fetal blood come into very close proximity (as little as 2–4 μm).

The fetal membranes

The fetal membranes contain the fetus throughout the pregnancy and eventually undergo programmed rupture during the first stage of labour. They consist of the fetal-facing amnion and maternal-facing chorion [5]. The amnion comprises five distinct layers. The innermost layer is the amniotic epithelium, which is in direct contact with the amniotic fluid on one side and a basement membrane on the other. The other layers consist of the compact layer, the fibroblast layer and the spongy or intermediate layer. The chorion consists of the reticular layer, a basement membrane and the trophoblast cell region, which at term firmly adheres to the maternal decidual tissue. Like the placenta, the fetal membranes play an integral role in fetal development and progression of pregnancy. In addition to autocrine regulatory activities, the membranes secrete substances both into the amniotic fluid, affecting amniotic fluid homeostasis, and towards the uterus, where they may influence maternal cellular physiology. The

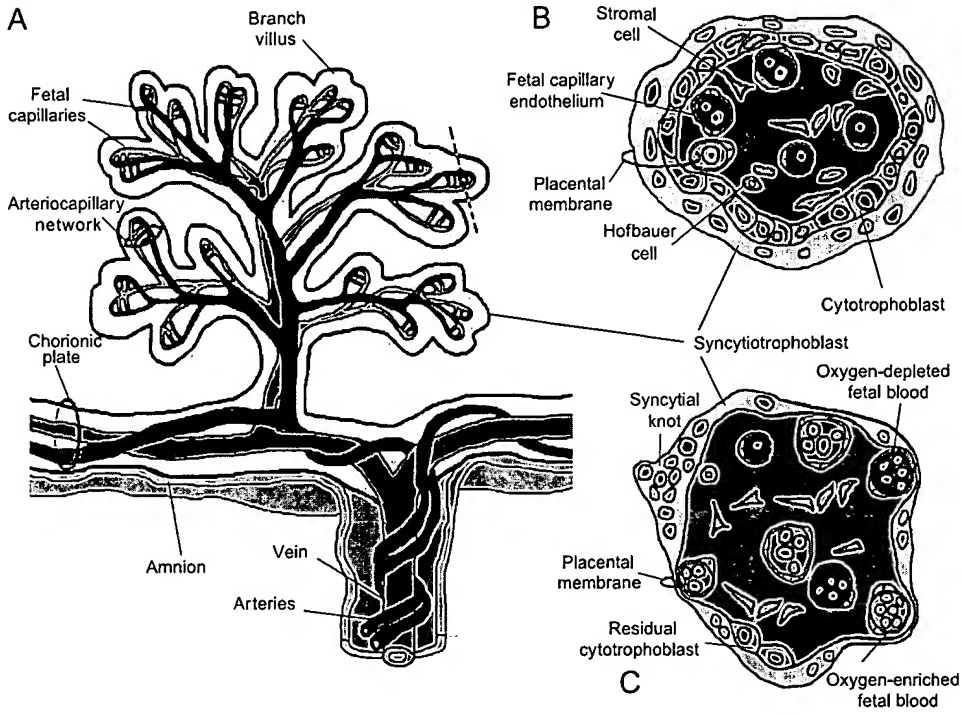

Figure 1 A representative drawing of the fetal placental circulation (A), in which the dotted line shows the position from which a drawing of a section through the chorionic villus at approximately 10 weeks (B) is taken. A section through the chorionic villus at full term is also shown (C).

membranes also play a protective role for the fetus against infection ascending the reproductive tract.

Implantation

The period for uterine receptivity for implantation is relatively short. Physiological preparation of the endometrium is modulated by cyclic secretion of 17β-estradiol and progesterone. These hormones regulate growth factors, cytokines and adhesion molecules that alter the endometrial surface and open the implantation window [3]. Other substances, such as fibronectin, close the window several days later. Prior to attachment to the endometrial epithelium, the zona pellucida surrounding the blastocyst is lost. Immediately after attachment, the trophoblast cell layer of the blastocyst proliferates rapidly and differentiates into an inner cytotrophoblastic layer and an outer multinucleated syncytiotrophoblastic mass. The syncytiotrophoblast extends into the endometrial epithelium and invades the connective tissue [6]. The blastocyst sinks beneath the endometrial surface, which is gradually repaired. Nourishment is obtained from the eroded maternal tissues and lacunar networks form within the syncytiotrophoblast [7]. Maternal blood moves in and out of these networks, thus establishing the uteroplacental circulation. Extensions of proliferating cytotrophoblast cells evaginate into the syncytiotrophoblast in various places. These extensions are the first stage in the development of the chorionic villi of the placenta [4].

The decidual reaction

Decidualisation of the endometrial stroma occurs as part of the normal menstrual cycle; however, in the event of pregnancy, decidual changes become more extensive. Glycogen and lipids accumulate in the cytoplasm of the cells causing them to enlarge and take on the appearance of the pale-staining decidual cells. The cellular and vascular changes of the endometrium as the blastocyst implants is referred to as the decidual reaction. The function of the decidua, however, is not certain. Rather than facilitating implantation and trophoblast migration, it has been suggested that the main role of the decidua is to restrain the inherently invasive trophoblast and control its migration [1]. This may be achieved by conversion of the motile invasive trophoblast cells into the static placental bed giant cells.

Trophoblast development

After successful implantation and initiation of placentation, trophoblast cells undergo extensive proliferation and differentiation. There are two main pathways by which trophoblast differentiation may occur, that is, villous and extravillous (Fig. 2). By days 13 to 14 of pregnancy, cytotrophoblast cells penetrate the layer of syncytiotrophoblast surrounding the early conceptus to form columns of extravillous cytotrophoblast cells. These contiguous cells form the cytotrophoblastic shell that is at the interface of the feto-maternal compartments [6]. Extravillous trophoblast cells invade the decidua and migrate so that they penetrate and remodel maternal blood vessels in the uterine decidua (endovascular trophoblast). This process produces dilated, compliant uterine arterioles that are unresponsive to maternal vasomotor control. Thus, the maternal blood supply to the placenta is promoted by this process and is, by term, about 30% of the mother's cardiac output, which itself has increased by 30–40% [8]. Trophoblast cells do not invade the decidual veins. Nevertheless, syncytial knots that detach from the chorionic villi into the intervillous space are deported into the maternal circulation via these veins [1].

Extravillous cytotrophoblast cells also invade interstitially (interstitial trophoblast). These invasive cells promote the circumferential expansion of the placental site and recruitment of maternal arterioles; allowing subsequent expansion of the villous region of the placenta below [6]. The full thickness of the uterine mucosa to the decidual–myometrial border has been extensively colonised by 8 weeks of pregnancy. Interstitial trophoblast cells become multinucleated and more rounded and form placental bed giant cells as they move deeper into the decidua [1]. These cells are regarded as the terminally differentiated end-point of the extravillous pathway (Fig. 2).

Extravillous cytotrophoblast cells at the tips of anchoring villi rapidly proliferate to form the cytotrophoblast cell columns. Cells distal in the column subsequently switch from an epithelial to a mesenchymal cell type, facilitating their migration into, and invasion of, the decidua and its vascula-

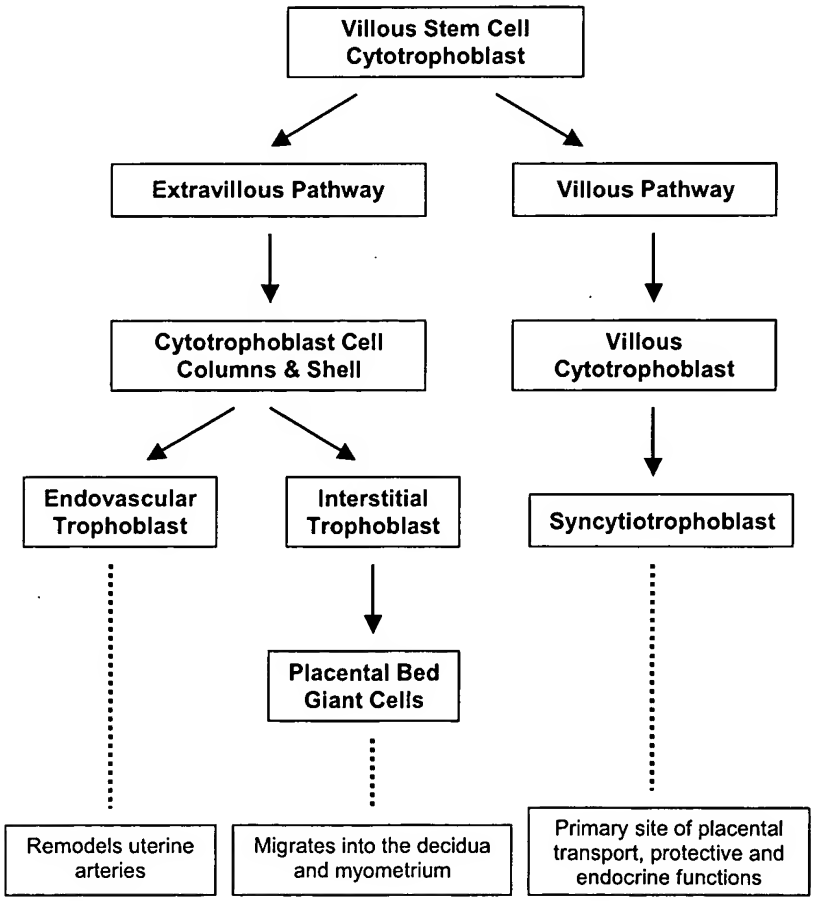

Figure 2 The pathways of trophoblast differentiation and function.

ture. This phenotypic switch is essential for migration of the cells [9]. Production and/or secretion of type IV collagenase, matrix metalloproteinases, β-glucuronidase, aminopeptidases, cathepsin B, urokinase-type plasminogen activator (uPA), uPA receptor and laminin by the extravillous cytotrophoblast cells enables their infiltration into the decidua by promoting degradation of extracellular matrix [10]. Insulin-like growth factor-II (IGF-II) may also be involved in this process, as it is abundantly expressed at the invading front [11] and induces cytotrophoblast migration in culture [12]. Counterbalancing the degradative activity that promotes invasion of the decidual extracellular matrix, extravillous cytotrophoblast cells also secrete plasminogen activator inhibitor (PAI) and tissue inhibitors of matrix metalloproteinases (TIMPs). These secretory activities appear to be regulated by autocrine and/or paracrine actions of growth factors, particularly, transforming growth factor (TGF)-β [13].

Villous (non-migratory) cytotrophoblast cells proliferate, differentiate and fuse to form the outer epithelial layer of the chorionic villi, the syncytiotrophobast (Fig. 2). The primary villi are formed by evaginations of syncytiotrophoblast with a cytotrophoblast core. Fetal mesenchyme grows into the cytotrophoblast to form the secondary villi and by the third week of gestation fetal capillaries develop within the villous mesenchyme forming the tertiary villi [6]. Initially, chorionic villi are found on the surface of the entire chorion but as the conceptus grows the decidua on the uterine luminal pole (decidua capsularis) atrophies, as does the villi apposed to it. This leaves the definitive placental villi in a discoid region [6]. As gestation progresses the chorionic villi grow and arborise (see below). Exchange takes place in the most part through the terminal villi that project into the intervillous space. It is the tips of villi that are attached to the basal plate (anchoring villi) that give rise to the invasive cytotrophoblast cell columns [6].

Paradoxically, during the first trimester of human pregnancy, the placenta differentiates and grows under a low oxygen environment. This is because uterine spiral arterioles are 'plugged' by endovascular cytotrophoblast cells and uterine blood flow to the conceptus is limited [14]. It is not until about 10–12 weeks of pregnancy that maternal blood begins to flow from the maternal spiral arteries into the intervillous space [15]. At this time, oxygen tension rises from <20 mm Hg (8 weeks) to >50 mm Hg at 12 weeks [16]. Exposure to a relatively hypoxic environment is thought to be an important regulator of cytotrophoblast function in early pregnancy. Indeed, it has been shown in vitro that proliferation and differentiation of human extravillous cytotrophoblasts is regulated by oxygen tension [17]. Evidence suggests that limited blood flow in first trimester is essential to pregnancy success. Assessment by Doppler ultrasound demonstrated that women who had premature onset of blood flow to the intervillous space had a higher incidence of miscarriage [18].

Blood vessel formation

The microvascular fetal capillary networks within the terminal villi are the culmination of the vascular tree of the placenta that originates with the macrovascular arteries and vein of the umbilical cord. The umbilical cord envelops a pair of arteries that carry deoxygenated blood and waste products to the placental villi, and a vein that carries oxygenated blood to the fetus. Without proper vascularisation, optimal growth and function of the placenta is not possible.

Vascularisation of the embryo and placenta commences at about 21 days post-conception, when villi initially undergo vasculogenesis and blood vessels are formed [19]. In the following phase, branching angiogenesis predominates. This involves the formation of new branches from pre-existing vessels and results in increased capillary density. During this period, there is a corresponding rise in end-diastolic blood flow velocity, most likely due to decreased fetoplacental vascular impedance and increased fetal blood pressure [20]. The steadily increasing metabolic demands of the growing fetus are matched by the increased blood flow and growth of the placenta [21]. The final phase of villous blood vessel growth occurs around the beginning of the third trimester (24–26 weeks). This period is characterised by the longitudinal growth of the capillaries, the characteristic looping and coiling of the capillaries and the formation of the terminal villi [22]. During this phase, non-branching angiogenesis predominates and exponential formation of the terminal villi ensues [2]. Terminal villus formation dramatically increases the surface area to volume ratio and, as described above, it is these villi that are the primary sites of gas and nutrient exchange [2]. Increased blood flow within the placenta is provided by the formation and growth of the placental vascular bed and regulated by changes in the dimensions and spatial arrangements of the vessels [23]. The important relationship between villous vascular-

isation and optimal embryonic/fetal growth is made evident by the association of common pregnancy disorders that involve poor fetal growth (e.g. fetal growth restriction and pre-eclampsia) and impaired fetal placental vascularisation [24].

Placental function

The main functions of the placenta can be categorised under the headings of transport and metabolism, protection and endocrine. The placenta acts to provide oxygen, water, carbohydrates, amino acids, lipids, vitamins, minerals and other nutrients to the fetus, whilst removing carbon dioxide and other waste products. It metabolises a number of substances and can release metabolic products into maternal and/or fetal circulations. It can help to protect the fetus against certain xenobiotic molecules, infections and maternal diseases. It also releases hormones into both the maternal and fetal circulations to affect pregnancy, metabolism, fetal growth, parturition and other functions. Many placental functions change during gestation, and there are many species differences in placental function. However, unless otherwise stated, it is specifically human placental function that is discussed below.

Transport and metabolism

Much of what is known about human placental transport is derived from studies of term placenta. However, there is increasing evidence that placental transport in early pregnancy may differ from that at term in many respects [25]. An important factor that may alter the expression of particular transporter proteins is oxygen tension and blood flow to the intervillous space. As described above, maternal blood flow to the intervillous space is only established from 10 to 12 weeks of gestation. During the first trimester prior to the onset of maternal blood flow to the intervillous space, nutrition is histiotrophic with trophoblast phagocytosis of endometrial glandular secretions including glycogen and a variety of glycoproteins [26]. After 10–12 weeks, maternal blood is in contact with the terminal villi of the placenta, and transfer of respiratory gases, nutrients and waste products occurs across the placental membrane. The placenta is not just a simple conduit for gases, nutrients and waste products, since it requires oxygen and nutrients for itself, and it produces metabolic products.

Transfer of respiratory gases

The placental membrane is highly permeable to respiratory gases. Thus rapid diffusion of oxygen can readily occur from maternal to fetal blood, and of carbon dioxide from fetal to maternal blood. Because diffusion of respiratory gases occurs so readily, a rate-limiting factor for their transfer can be blood flow to and from the site of transfer. Interestingly, fetal haemoglobin has a higher affinity for oxygen and a lower affinity for carbon dioxide than maternal haemoglobin. This will therefore favour transfer of oxygen to the fetus and carbon dioxide to the mother.

Transport and metabolism of carbohydrates

Glucose is the main carbohydrate transported across the placenta from mother to fetus. It is a primary source of energy for the fetus, and it can also partake in a number of anabolic processes. As the fetus is capable of very little gluconeogenesis, this glucose must be derived from the maternal circulation. Transport of glucose across the placenta is generally via protein-mediated facilitated diffusion, and a number of glucose transporters (GLUTs) are involved.

Uptake of maternal glucose occurs initially across the microvillous membrane of the syncytiotrophoblast. Once inside the syncytiotrophoblast cytoplasm, glucose can be transported out of the syncytiotrophoblast, e.g. via the basement membrane towards the fetal capillary endothelial cells, which also have membrane-located glucose transporters. However, the rate-limiting step for maternal–fetal glucose transfer is thought to be within the syncytiotrophoblast [27]. In the syncytiotrophoblast, glucose can be converted into glucose-6-phosphate or placental glycogen. Glucose-6-phosphate, in turn, can be utilised via aerobic or anaerobic respiration or via the pentose phosphate pathway. Human placental glucose transport is stereo-selective (for D-glucose), and placental transfer of fructose occurs at a much lower rate than that of glucose.

The facilitative glucose carrier GLUT1 has been located at term both in the maternal blood-facing and fetal capillary-facing membranes of the placental tissues, and is thought to be responsible for a major component of glucose transport across term placenta [28]. At term, GLUT3 is localised to the endothelial cells lining the fetal capillaries and is thought to be important for regulating glucose levels between these cells and fetal blood [28]. GLUT4, an insulin-responsive glucose transporter, is present in placental stromal cells and may be

important for transporting glucose and conversion to glycogen in these cells in response to insulin in the fetal circulation [29]. GLUT8 has been found to be expressed in human placenta at term, but may be less important in early pregnancy [30]. At term, immunohistochemistry has shown that GLUT12 staining was virtually completely absent from the syncytiotrophoblast and was found predominantly in villous vessel smooth muscle cells and villous stromal cells [31].

There are differences in cellular distribution of GLUTs between first trimester and term placentas that suggests differences in function in early and late pregnancy. Table 1 summarises these differences. While GLUT1 may be important for maternal–fetal glucose transport throughout pregnancy, GLUTs 3, 4 and 12 may only be important for this function in first trimester.

The human placenta produces large amounts of lactate, and lactate is also transported by the placenta. The human fetus may also be a net lactate producer, and the placenta can play a role in removing lactate from the fetus [34].

Transport and metabolism of amino acids

Amino acids are required by the fetus for protein synthesis, but they can also be metabolised by the fetus. There are over 20 amino acids found in plasma, some of which are not synthesised by human tissues ("essential" amino acids), and some of which can be synthesised, e.g. from glycolytic and citric acid cycle intermediates. Transport of amino acids to the fetus during pregnancy occurs via the microvillous and basal membranes of the syncytiotrophoblast. The ratio of most amino acids in fetal compared with maternal plasma is generally greater than 1 (typically between 1 and 4) [35], indicating active, i.e. energy-requiring, transport of amino acids from mother to fetus. There are many isoforms of amino acid transporters, and several families to which these isoforms belong. The expression of different isoforms can vary between and within different tissues.

Amino acid transporters can be categorised as heterodimeric or monomeric. Heterodimeric transporters consist of a heavy chain linked to one of a series of at least seven light chains. A number of heterodimeric amino acid transporters are thought to be expressed in placenta [36], including isoforms of the following transporter families: system L (that can transport a number of neutral amino acids); system y^+L (that can mediate the exchange of cationic amino acids for sodium and neutral amino acids); and system $b^{0,+}$ (that can mediate sodium-independent transport of cationic amino acids and cystine).

As with heterodimeric amino acid transporters, monomeric transporters belong to a number of families, each with a variety of isoforms. The main families found to be expressed in human placenta [36] include the following: system y^+ (a high capacity sodium-independent transporter of cationic amino acids); system X_{AG}^- (a transporter of the anionic amino acids glutamate and aspartate that is thought to utilise ionic gradients for active transport); system ASC (a sodium-dependent transporter of short chain neutral amino acids such as alanine, serine and cysteine); and system A (a sodium-dependent transporter of neutral amino acids).

Transport and metabolism of lipids

Lipids include free fatty acids, triacylglycerols, phospholipids, glycolipids, sphingolipids, cholesterol, cholesterol esters, fat-soluble vitamins, and a variety of other compounds. Many lipids are bound to proteins within plasma. For example, free fatty acids bind to serum albumin, whereas phospholipids, cholesterol and triacylglycerol form a number of different types of lipoprotein complexes. The maternal surface of the placenta contains lipoprotein lipase, which can release free fatty acids from the lipoprotein complexes circulating in maternal plasma. Both free fatty acids and glycerol (but not triacylglycerols) can readily cross the membranes of the placental syncytiotrophoblast. They can do so by simple diffusion, as they

Table 1 Distribution of GLUT isoforms in human placenta in first trimester and term

GLUT isoform	First trimester	Term	References
GLUT1	Syncytiotrophoblast, villous cytotrophoblast, vascular smooth muscle, endothelium, stromal cells	Syncytiotrophoblast, villous cytotrophoblast, vascular smooth muscle, endothelium, stromal cells	[27,28]
GLUT3	Extravillous cytotrophoblast, villous cytotrophoblast	Endothelium	[27,28,32]
GLUT4	Syncytiotrophoblast	Stromal cells	[27,29,33]
GLUT12	Syncytiotrophoblast, extravillous cytotrophoblast, villous cytotrophoblast	Vascular smooth muscle, stromal cells	[27,31]

are lipophilic. However, they can also cross via the action of membrane-bound and cytosolic fatty acid binding proteins [37]. These proteins are important in determining the direction and amount of net flux of fatty acids. The placenta is able to preferentially transport long chain polyunsaturated fatty acids [38], and the fetal blood is enriched in these compounds compared with maternal blood.

Once fatty acids reach the cytoplasm of the placental trophoblast, they can bind to cytosolic binding proteins, they can be transported out of the trophoblast, or alternatively they can be oxidised or esterified. Placental microsomes contain the enzymes necessary for the synthesis of glycolipids from glycerol-3-phosphate, free fatty acids and other precursors [39]. Also, cultured human trophoblast has been shown to readily synthesise oleic, palmitic and palmitoleic acids, and to have a limited capacity for synthesis of stearic, myristic and lauric acids [40]. Although the placenta can synthesise cholesterol, under normal circumstances cholesterol is derived from maternal blood via an interaction of circulating low-density lipoproteins (LDLs) with LDL receptors on the microvillous membrane of the syncytiotrophoblast, followed by internalisation of LDLs by receptor-mediated endocytosis.

The liver and biliary system are responsible for the biotransformation and elimination of bile acids, biliary pigments and many lipid-soluble exogenous compounds. However, the fetal liver is immature, and the placenta subsumes many of the roles of the adult liver. For example, the placental trophoblast contains enzymes and transporting proteins that are involved in the handling of bile acids, biliary pigments and xenobiotics [41].

Transfer of water, inorganic ions, minerals and vitamins

Water transfer across the placenta is dependent upon hydrostatic and osmotic pressure. It is presumed to move across the placenta passively, and its transfer may be facilitated by a water channel-forming integral protein expressed in the trophoblast [42].

Sodium and chloride levels in fetal and maternal blood are similar, whereas potassium, calcium and phosphate levels are higher in fetal blood [43]. Potassium, magnesium, calcium and phosphate are all transported across the placenta actively, whereas at least in some species sodium and chloride transfer may occur passively [42]. The situation is quite complex, however, as there are many active ion-transporting systems in the placenta, including Na/K ATPase, Ca ATPase, Na/H exchangers [44], and many others. Also, ion transport can be affected by proteins such as sodium-dependent amino acid transporters discussed above.

Vitamins are transferred from the maternal to the fetal circulations, as are many minerals. For example, iron dissociates from transferrin at the placental interface, and is transported across the placenta.

Endocrine functions of the placenta

The placenta is devoid of nerves, and therefore any communication between it and the mother and/or fetus would normally occur via blood-borne substances. Substances are also produced by the placenta that can play a localised role, e.g. in the uterus or within the placenta itself.

Endocrine, paracrine and/or autocrine factors that are produced by the placenta include oestrogens (produced in conjunction with the fetal adrenal gland and possibly fetal liver), progesterone, chorionic gonadotrophin, placental lactogen, placental growth hormone, a number of growth factors (including epidermal growth factor, insulin-like growth factors I and II, platelet-derived growth factor), cytokines, chemokines, eicosanoids and related compounds, vasoactive autacoids, pregnancy-associated proteins of placental origin, corticotrophin-releasing hormone, gonadotrophin-releasing hormone, thyrotrophin-releasing hormone and many others. Some of these are briefly discussed below.

Progesterone is produced by the human placenta and is released into both maternal and fetal circulations. Progesterone inhibits uterine contraction. It suppresses oestrus and release of luteinizing hormone from the pituitary gland. The corpus luteum also produces progesterone, but by about the 9th week of pregnancy it has atrophied, and then the placenta is responsible for the production of most of the circulating progesterone. At around this time, the placenta also becomes the main source of circulating oestrogens, which include oestrone, oestradiol and oestriol. Oestrogens act as specialised growth hormones for the mother's reproductive organs, including breasts, uterus, cervix and vagina [39]. Conjugation of oestrogens (e.g. with sulphate or glucuronide) occurs within the fetal circulation, and may help to protect the fetus from high levels of free oestrogens.

Human chorionic gonadotrophin (hCG) is a dimeric glycoprotein that is produced by the trophoblast and secreted predominantly into the mater-

nal circulation. It is produced mainly in early pregnancy with peak levels at about 8 weeks, falling to low levels from about 12 weeks, but with a rise again in late pregnancy. It may help prolong the life of the corpus luteum in early pregnancy. Cytotrophoblast cell fusion and the functional differentiation of villous trophoblast are stimulated by hCG, as well as by estradiol and glucocorticoids [45].

Human placental lactogen has homology with both human growth hormone and prolactin. It is synthesised by the syncytiotrophoblast and released into both maternal and fetal circulations. In the fetus, human placental lactogen acts to modulate embryonic development, regulate intermediary metabolism and stimulate the production of insulin-like growth factors, insulin, adrenocortical hormones and pulmonary surfactant [46]. It may also be involved in angiogensis [47].

Placental growth hormone differs from pituitary growth hormone by 13 amino acids. It is secreted by the placenta into the maternal circulation and may play a role in maternal adjustment to pregnancy, control of maternal insulin-like growth factor I (IGF-I) levels, and placental development via an autocrine or paracrine mechanism [48]. Both human placental growth hormone and human placental lactogen act to stimulate maternal IGF production and modulate intermediary metabolism, resulting in an increase in the availability of glucose and amino acids to the fetus [46].

Insulin-like growth factors I and II (IGF-I and -II) are produced by fetal tissues and play an important role in feto-placental growth throughout gestation. Both *Igf1 and 2* genes are expressed in human placenta, and the role of IGF-I and -II in feto-placental and fetal growth have been recently reviewed [49].

The placenta produces small amounts of chorionic thyrotropin and corticotropin that are released into the maternal blood stream and may help modulate maternal metabolism and other physiological functions.

The placenta and extraplacental membranes produce a large number of cytokines, chemokines, eicosanoids and related factors, and some of these may be involved in parturition [50]. Eicosanoids may also be involved in control of blood flow in the placenta, along with many other locally produced autacoids [51]. Indeed, there are numerous vasoactive autacoids that are produced by the placenta including endothelins [52], adrenomedullin [53], nitric oxide [54] and many others.

There are many pregnancy-associated proteins of placental origin, and not all of these have been well studied. One that has been studied is pregnancy-associated plasma protein A (PAPP-A), which is produced by the placenta and belongs to the metzincin superfamily of metalloproteinases. It is an insulin-like growth factor binding protein-4 (IGFBP-4) proteinase, and its levels may be reduced in first trimester when a fetus with Down's syndrome is present [55].

The placenta produces large amounts of acetylcholine [56]. Although the functions of placental acetylcholine are not clear, it has been postulated that non-neuronal acetylcholine may play a role in cell proliferation, differentiation, organization of the cytoskeleton and the cell–cell contact, cell migration and immune functions [57].

Protective functions of the placenta

The placenta can act to protect the fetus from certain xenobiotics that could be circulating in maternal blood. Many small xenobiotic molecules can cross the placenta by simple diffusion via transcellular or paracellular routes. Alternatively, some xenobiotics can be transported across the placenta by one or more of the large number of placental transport systems, many of which are not completely specific for the endogenous transported molecule(s). However, there are a number of protective features of the human placenta, which can help reduce placental transfer of potentially toxic substances. These features include export pumps in the maternal-facing membrane of the syncytiotrophoblast, including multidrug resistance protein 1 (MDR1), several members of the multidrug resistance-associated protein (MRP) family, placenta-specific ATP-binding cassette proteins (ABCP), breast cancer resistance protein (BCRP) and mitoxantrone resistance-associated protein (MXR) [41]. In addition, the placenta contains a number of cytochrome *P*450 enzymes that can metabolise drugs and other xenobiotics, together with other phase I and phase II xenobiotic-metabolising enzymes [58]. However, although the placenta can help reduce the exposure of the fetus to some xenobiotic substances, there are many that can cross the placenta and have teratogenic effects, including alcohol, thalidomide, many anticonvulsants, lithium, warfarin, isotretinoin and numerous others.

Although most proteins do not readily cross the placenta, some are transported across the placenta by pinocytosis, including maternal antibodies mainly of the immunoglobulin G class. Such antibodies help provide passive immunity in the newborn baby. There has been considerable debate about how the trophoblast tissue of the human placenta resists immunological rejection from the maternal immune

system present in the adjacent uterine decidua, but no consensus has emerged [59].

The placenta generally forms a barrier against transmission of many bacteria from mother to fetus. However, some bacteria, some protozoa, and a number of viruses can be transmitted across the placenta. For example, although the majority of human immunodeficiency virus (HIV) infection is transmitted from mother to baby around the time of birth, it is estimated that in about 1.5–2% of pregnancies in HIV-positive mothers, transplacental HIV transfer may occur, e.g. via HIV binding to lectins expressed by the placenta with subsequent viral absorption [60]. Other viruses that can infect the fetus include cytomegalovirus, rubella, polio, varicella, variola and coxsackie viruses. The bacterium that causes syphilis can also be transmitted across the placenta, as can the protozoal parasite that causes toxoplasmosis. It has also been postulated that viral infection of trophoblast may be related to poor pregnancy outcomes [61].

In summary, the placenta is the physical and functional connection between the mother and the developing embryo/fetus. Within the placenta, growth and function are precisely regulated and coordinated to ensure the exchange of nutrients and waste products between the maternal and fetal circulatory systems operates at maximal efficiency. The placenta is also intimately associated with the tissues and vasculature of the maternal decidua. These interactions must be carefully orchestrated to allow subtle but necessary changes to the maternal circulation in order to cope with the increasing metabolic demands of the fetus as it develops.

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Maternal Insulin-Like Growth Factors-I and -II Act via Different Pathways to Promote Fetal Growth

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The placenta transports substrates and wastes between the maternal and fetal circulations. In mice, placental IGF-II is essential for normal placental development and function but, in other mammalian species, maternal circulating IGF-II is substantial and may contribute. Maternal circulating IGFs increase in early pregnancy, and early treatment of guinea pigs with either IGF-I or IGF-II increases placental and fetal weights by mid-gestation. We now show that these effects persist to enhance placental development and fetal growth and survival near term. Pregnant guinea pigs were infused with IGF-I, IGF-II (both 1 mg/kg·d), or vehicle sc from d 20–38 of pregnancy and killed on d 62 (term = 69 d). IGF-II, but not IGF-I, increased the mid-sagittal area and volume of placenta devoted to exchange by approximately 30%, the total volume of trophoblast and maternal blood spaces within the placental exchange region (+29% and +46%, respectively), and the total surface area of placenta for exchange by 39%. Both IGFs reduced resorptions, and IGF-II increased the number of viable fetuses by 26%. Both IGFs increased fetal weight by 11–17% and fetal circulating amino acid concentrations. IGF-I, but not IGF-II, reduced maternal adipose depot weights by approximately 30%. In conclusion, increased maternal IGF-II abundance in early pregnancy promotes fetal growth and viability near term by increasing placental structural and functional capacity, whereas IGF-I appears to divert nutrients from the mother to the conceptus. This suggests major and complementary roles in placental and fetal growth for increased circulating IGFs in early to mid-pregnancy. (*Endocrinology* 147: 0000–0000, 2006)

THE PLACENTA IS a multifunctional organ that forms the interface between the fetal and maternal circulations. It is essential for fetal growth as it supplies the developing fetus with oxygen and nutrients, transporting them from the mother into the umbilical circulation. Abnormalities in placental structural development can impair placental function, reducing substrate supply to the fetus, and may result in intrauterine growth restriction (1). It is estimated that placental dysfunction accounts for 70–80% of growth-restricted newborns (2), currently affecting 6% of pregnancies in developed countries (3) and up to 40% in developing countries (4). Intrauterine growth restriction is associated with perinatal morbidity and mortality (5, 6) and increases the risk of poor health in childhood and adult life (7). In addition, impaired placental trophoblast invasion of the maternal uterine vasculature and/or poor placental function are implicated in other major pregnancy complications, such as miscarriage (8), preeclampsia (1), placental abruption (9), and preterm labor (10, 11). Therefore, it is imperative that we understand the factors essential for regulating placental functional development to identify causes of such diseases and as a basis for the development of therapeutics.

The IGF-I and -II have been implicated in placental structural and functional development. *Igf2* overexpression in mice causes placental and fetal overgrowth (12), whereas *Igf2* gene deletion reduces placental weight by 17% on d 13.5 and 25% on d 16.5 of gestation, with a fetal weight reduction of 40% from d 16.5 (term = 19 d) (13, 14). In addition, placental amino acid transporter expression is altered by *Igf2* deficiency in mice (15). Ablation of the placental-specific *Igf2* promoter (P0) in mice reduces placental weight and adversely affects placental structural differentiation and transport capacity, with reduced fetal weight evident 2 d later (16, 17). The latter reduction in fetal weight was comparable to that induced by global *Igf2* gene ablation, suggesting that the effects of *Igf2* deficiency on fetal growth are mediated by actions on the placenta in mice.

In contrast, *Igf1* gene ablation in mice does not alter placental weight but reduces fetal weight, indicating that IGF-I is important in the fetus (14, 18). IGF-I may modulate placental nutrient capacity because IGF-I administration to pregnant rats, or increased endogenous expression in pregnant mice, increases the weight of the fetus but not that of the placenta (19). IGF-I stimulates glucose and amino acid uptake in cultured human placental trophoblasts (20–22) and promotes placental nutrient uptake and metabolism when infused into fetal sheep (23–25). Moreover, exposure to IGF-I inhibits release of vasoconstrictors, such as thromboxane B2 and prostaglandin F2 α, in human term placental explants (26), which may increase placental blood flow and delivery of nutrients for the growth of the fetus.

The placenta is exposed to IGFs from multiple sources, including those produced locally and those circulating within the fetus and mother. Maternally derived IGFs may have a major influence on placental development, particularly in women and in guinea pigs where circulating IGFs are substantial (27, 28). Indeed, the IGF axis in guinea pigs is very similar to that of humans (29), whereas rats and mice do not

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Abbreviation: IGFBP, IGF binding protein.

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have circulating IGF-II postnatally. The placenta in guinea pigs is more similar to the human placenta than that of other nonprimate species being hemomonochorial, although it is labyrinthine rather than villous in structure. The guinea pig placenta is comprised of a labyrinth, which contains both fetal capillaries and maternal blood sinuses and provides the means for exchange between the two circulations and an interlobium that is comprised of syncytiotrophoblast and maternal blood sinuses, and is the site where much of the metabolic activity of the placenta is thought to occur (30). In the human placenta, exchange and endocrine functions are performed in the placental villi (31). In addition, placental trophoblast cells in guinea pigs are highly invasive and, like those in humans, engage in interstitial and endovascular invasion of the decidua. They remodel the uterine spiral arterioles to permit the large increase in blood flow to the placenta (32, 33) that is essential for placental growth and subsequent function and therefore fetal growth.

In the guinea pig, major structural determinants of placental function are strongly predicted by maternal IGF-II concentration in mid-pregnancy and by maternal IGF-I in late pregnancy (34, 35). Furthermore, in this species, food restriction reduces maternal plasma IGF concentrations (36) that correlate with delayed structural and functional maturation of the placenta and with reduced fetal growth (34, 35, 37). The structural defects in the placenta of food-restricted guinea pigs are similar to those seen in placentas from women with preeclampsia (34). In addition, reduced maternal plasma IGF-I in pregnant women is associated with placental dysfunction and small-for-gestational-age (38, 39) or growth-restricted infants (40).

Consistent with these adaptive changes in maternal IGFs regulating placental development, maternal supplementation with IGF-I or IGF-II in early to mid-pregnancy in the guinea pig increases placental and fetal weights by mid gestation (41). Therefore, we suggest that the increased maternal production of both IGFs in early pregnancy is an important adaptation to pregnancy, which promotes placental functional development and consequently fetal growth. Whether anabolic effects of an increased abundance of maternal IGFs in early pregnancy on the placenta would persist into late gestation and affect the fetus is currently unknown. Therefore, the aim of this study was to determine the effects of maternal IGF-I and -II supplementation in early to mid-pregnancy on placental development and fetal growth and viability near term.

Materials and Methods

Animals

This study was approved by the University of Adelaide, Animal Ethics Committee. Virgin guinea pigs (IMVS colored strain, approximately 500 g, 3–4 months old) were housed individually in the University of Adelaide Medical School Animal House. Guinea pigs were provided with food and water *ad libitum*. Females were examined daily for estrus indicated by a ruptured vaginal membrane (complete estrous cycle lasts approximately 15 d) and mated naturally with a male. The day a copulatory plug was observed was designated as d 1 of pregnancy. From 2 wk before mating, body weight was monitored three times weekly. Females were assigned to three groups of similar mean weight at mating.

On d 20 of pregnancy (term 69–70 d), females were anesthetized with atropine sulfate (0.05 mg/kg, sc; Apex Laboratories, Kariong, Australia), xylazine hydrochloride (4 mg/kg, im; Troy Laboratories, Smithfield, Australia), ketamine hydrochloride (25 mg/kg, ip; Troy Laboratories) AQ: A and administered local analgesia with lignocaine hydrochloride (Troy Laboratories). A 200-μl mini osmotic pump (Alzet 2002; Alzet, Cupertino, CA) was surgically inserted sc. Minipumps had previously been prepared to deliver vehicle (0.1 M acetic acid) (n = 7) or 1 mg/kg·d IGF-II (n = 7) or IGF-I (n = 9) (human recombinant protein; GroPep Pty. Ltd., Adelaide, Australia) for 18 d at a flow rate of 0.51 μl/h.

On d 62 of pregnancy, guinea pigs were killed by overdose of sodium pentobarbitone (Lethobarb; Virbac, Peakhurst, Australia). Viable and AQ: B resorbing implantation sites were counted and the uterus and its contents, viable fetuses, and placentae were weighed. Fetal biparietal diameter, abdominal circumference, and crown-to-rump length were measured. A 3-mm mid-sagittal placental slice was fixed in 4% paraformaldehyde for structural analysis. Analyses of body composition were performed on the mothers and all fetuses to determine the absolute and relative weights of adrenals, kidneys, pancreas, liver, spleen, heart, brain, lungs, gastrointestinal tract, reproductive tract, biceps, triceps, gastrocnemius and soleus muscles and retroperitoneal, perirenal, and interscapular adipose tissues. Skin and carcass weights of the dams and carcass weight of the fetuses were also recorded.

Measurement of maternal circulating IGF-I, IGF-II, and IGF binding proteins (IGFBPs)

In an additional cohort of guinea pigs (vehicle, n = 5; IGF-I, n = 5; IGF-II, n = 3), mothers were killed on d 35 of pregnancy, while the minipumps were still active by overdose of sodium pentobarbitone. Maternal blood was collected by cardiac puncture and centrifuged at 2500 rpm for 15 min at 4 C, then plasma was recovered and stored at −20 C.

Plasma IGF-I and IGF-II proteins were dissociated from their binding proteins (IGFBPs) by size exclusion high pressure liquid chromatography performed at pH 2.5, as previously described (42, 43). From each acidified plasma sample, four fractions were eluted from the column, and fraction 1, which contained only IGFBPs, and fraction 3, which contained only the IGFs, were collected for later analysis. The IGF fraction 3 was analyzed by specific RIAs for IGF-I and IGF-II concentrations as previously described (42, 44).

Recombinant human IGF-I and IGF-II (GroPep Pty. Ltd.) were used as standards and for preparation of radiolabeled ligands. IGF-I was measured by RIA using rabbit antihuman IGF-I (MAC Ab 89/1; GroPep Pty. Ltd.) at a final dilution of 1/60,000 and a monoclonal mouse antirat IGF-II antibody (kind gift from Dr. K. Nishikawa, Kanaza Medical University, Ishakawa, Japan) was used at a final concentration of 1/500 to measure IGF-II by RIA. Cross-reactivity of IGF-II in the IGF-I RIA was less than 1% (44) and that of IGF-I in the IGF-II RIA was less than 2.5% (45). Both IGF-I and IGF-II amino acid sequences are remarkably conserved across species. Guinea pig IGF-I and IGF-II have previously been shown to have 100% amino acid sequence identity to those of human (46, 47), whereas guinea pig IGF-II has only one amino acid different to that of the rat (48). We have previously reported that the recoveries of IGF-I and IGF-II are more than 95% for these assays (28). The minimal detectable concentrations of IGF-I and IGF-II were 6.64 and 9.48 ng/ml, respectively. The samples were analyzed in a single RIA, where the mean intraassay coefficients of variation were 3.7 and 5.6% for IGF-I and IGF-II RIAs, respectively.

The total IGFBP binding capacity in the maternal circulation was indirectly measured as the interference of the IGFBPs in fraction 1 in the IGF-I RIA, as previously described (42). The ratio of IGFs to IGFBPs provided an index of IGF bioavailability in the maternal circulation.

Placental histology

Mid-sagittal slices of placentae that had been fixed in 4% paraformaldehyde overnight were washed in 1% PBS, dehydrated, and embedded in paraffin wax, then 5-μm sections were stained with Masson's Trichrome (49). From each dam, one to three placentae were randomly selected for histological assessment. The cross-sectional areas of the placental interlobium (germinative region) and labyrinth (exchange region) were measured in complete mid-sagittal sections using an Olympus BH-2 microscope with ×2 objective and ×3.3 ocular lenses and video

image analysis software (Video Pro; Leading Edge, Adelaide, Australia). The proportion (percentage) of each region in the placenta was then estimated by dividing the cross-sectional area of that region by the total mid-sagittal cross-sectional area of the placenta. An estimate of the volume of these regions was then calculated by multiplying their proportion by total placental weight.

Structure of the placental exchange region (labyrinth)

To distinguish cell types within the placental labyrinth, mid-sagittal sections of placenta were double-labeled with mouse antibodies to human vimentin (3B4; Dako, Denmark) and human pan cytokeratin (C2562; Sigma) to identify fetal capillaries and trophoblast, respectively, and then stained with eosin to aid the identification of maternal blood spaces. This employed a triple layer technique for each antibody, performed sequentially. Sections were deparaffinized and brought to water. For antigen retrieval, sections were incubated at 37 C for 15 min in 0.03% protease (Sigma). Endogenous peroxidase activity was quenched by incubating with 3% hydrogen peroxide in water for 30 min. Sections were then washed in three changes of PBS for 5 min each and blocked for nonspecific binding with serum-free protein block (Dako) for 10 min without washing. 3B4 antibody diluted 1:50 with 10% normal guinea pig serum and 1% BSA was applied first and incubated overnight in a humidified chamber at room temperature. Sections were washed as above, and biotinylated goat antimouse IgG secondary antibody (Dako) was applied for 30 min, followed by washing. Streptavidin conjugated to horseradish-peroxidase (Rockland Immunochemicals, Gilbertsville, PA) was applied for 40 min, then sections were washed as above. 3B4 binding was visualized by incubating with diaminobenzidine with 2% ammonium nickel (II) sulfate (Sigma) to form a black precipitate. The process was then repeated for the second primary antibody (C2562) diluted 1:50 with PBS, 10% normal guinea pig serum, and 1% BSA, but nickel was omitted from the chromogen, leaving a brown precipitate. Negative controls used irrelevant mouse IgG in place of the primary antibodies or the primary antibody diluent on its own.

The placental labyrinth was then morphometrically analyzed, as previously described (34). Briefly, the proportions (volume density) and volumes of the labyrinthine placental components were quantitated by point counting on 10 nonoverlapping fields with random systematic sampling using an Olympus BH-2 microscope with ×20 objective and ×3.3 ocular lenses. The weight of each component was estimated by multiplying the volume density by the weight of the placental labyrinth. The surface area per gram of placental labyrinth was quantitated using intercept counting and the total surface area of syncytiotrophoblast for exchange and arithmetic mean trophoblast thickness (the layer through which substrate exchange occurs) were calculated as previously described (34).

Protein localization of IGF receptors in the placenta on d 35 of pregnancy

To determine that the placenta expressed the type 1 and 2 IGF receptors at the time of treatment we localized them in placental sections from the cohort of guinea pigs that were killed on d 35 of pregnancy in which circulating IGFs had been quantified. Mid-sagittal slices of placentae were immuno-labeled with rabbit antibodies raised against human IGF1R (N-20, diluted 1:20; Santa Cruz Biotechnology, Santa Cruz, CA) and IGF2R (a kind gift from Dr. Carolyn Scott, University of Western Australia, Perth, Australia; diluted 1:100). This employed a triple layer technique for each antibody performed on serial placental sections, as described above. Negative controls used irrelevant mouse IgG in place of the primary antibodies or the primary antibody diluent on its own.

Plasma metabolite and hormone concentrations

Maternal and fetal plasma glucose (glucose HK assay kit; Roche), free-fatty acids (WAKO Nefa C free fatty acid kit; NovoChem), cholesterol (cholesterol CHOD-PAP assay kit; Roche), and triglycerides (triglycerides assay kit; Roche) were quantified with enzymatic assay kits using a COBAS Mira automated centrifugal analyzer (Roche Diagnostic Systems). Maternal and fetal plasma α-amino nitrogen concentrations were determined using the β-naphthoquinone sulfonate colorimetric assay as previously described (50). Maternal plasma estradiol (Ultra-Sensitive Estradiol; DSL) and progesterone concentrations (progesterone assay kit; DSL) were quantified with RIA kits.

Statistics

To assess differences in fetal weight distribution between treatments, χ^2 tests were performed using Microsoft Excel. All other data were analyzed using SPSS version 13 (SPSS, Chicago, IL). To assess differences in maternal weight gain, repeated measured ANOVA with Bonferroni *post hoc* tests were performed. To assess differences in maternal body composition, general linear model univariate ANOVA with Bonferroni *post hoc* tests were performed. To assess differences in fetal band placental parameters, linear mixed model repeated measures ANOVA with Bonferroni *post hoc* tests were performed with the mother as a subject and the fetus or placenta as the repeated measure. The number of viable pups per litter were used as a covariate when required. Data are expressed as mean ± SEM or estimated marginal mean ± SEM as required. Data were considered statistically significant when $P < 0.05$.

Results

Exogenous maternal IGF treatment increases maternal plasma IGF-I and IGF-II

To determine the concentration of IGFs we achieved in the maternal circulation in response to this treatment, an additional cohort of guinea pigs was killed on d 35 of pregnancy, while the minipumps were still active. Exogenous IGF-I increased maternal plasma IGF-I by 340% ($P = 0.001$) and reduced that of IGF-II by 45% ($P = 0.008$; Fig. 1). Exogenous IGF-II did not alter plasma IGF-I concentrations but increased plasma IGF-II by 240% ($P = 0.004$; Fig. 1). In addition, the total apparent IGFBP activity in maternal plasma was not altered by exogenous IGF. Maternal IGF-I treatment increased the ratio of IGF-I to IGFBPs in plasma by 230% ($P = 0.004$), whereas IGF-II increased the ratio of IGF-II to IGFBPs in plasma by 125% ($P = 0.04$; Fig. 1).

IGF receptor proteins are expressed by the guinea pig placenta during the treatment

To establish that IGF1R and IGF2R are expressed by the guinea pig placenta during the IGF treatment, immunolabeling was performed on guinea pig placenta recovered from vehicle-treated mothers killed on d 35 of pregnancy (Fig. 2). IGF1R and IGF2R were ubiquitously expressed by the guinea pig placenta, with profuse cytoplasmic staining observed in trophoblast and fetal endothelium of the labyrinth and trophoblast of the interlobium (Fig. 2, A and C). Both IGF receptor proteins were concentrated on the apical surface of trophoblast within large maternal blood sinusoids and within maternal blood spaces (Fig. 2, B and D).

Exogenous maternal IGF-II, but not IGF-I, enhances development of the placental exchange region (labyrinth)

IGF treatment in early to mid-pregnancy did not alter placental weight in late gestation (Table 1). Exogenous IGF-II increased placental labyrinthine cross-sectional area by 28% ($P = 0.005$) but not that of the interlobium (Fig. 3, A–C, and Table 1). The ratio of labyrinth to interlobium was increased by IGF-II (+37%, $P = 0.054$). IGF-II increased the proportion of the placenta comprised of labyrinth (+9%, $P = 0.0003$) and reduced that composed of the interlobium (−24%, $P = 0.0003$) (Table 1). IGF-II also increased the volume of pla-

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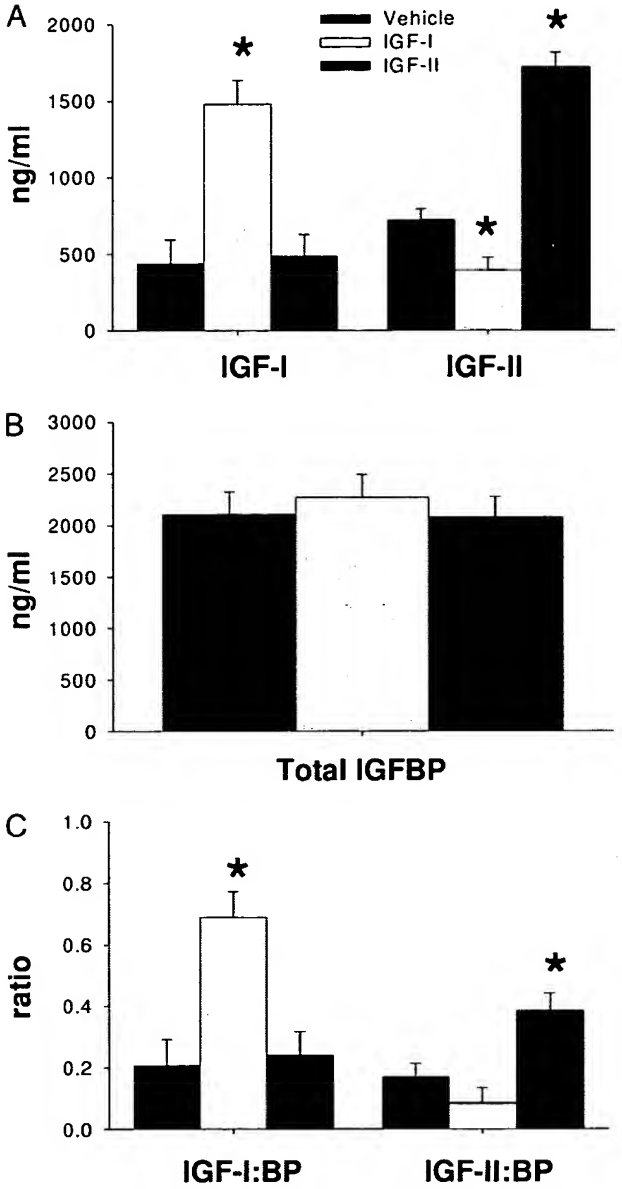

FIG. 1. The effect of exogenous maternal IGFs on maternal circulating IGF-I, IGF-II (A), and total IGFBP (B) concentrations and bioavailability of IGFs in the circulation indicated by IGF to IGFBP ratios (C) during treatment on d 35 of pregnancy. Data are from three to six mothers per treatment, and values are expressed as means ± SEM. *Asterisks* denote a statistically significant difference compared with the vehicle group, $P < 0.034$.

cental labyrinth (+28%, $P = 0.027$) but did not alter that of the interlobium (Table 1). Maternal IGF-I treatment did not alter any placental parameter (Table 1).

To examine placental labyrinthine development in response to earlier maternal IGFs in more detail, structural correlates of placental function were quantified. Maternal IGF treatment did not alter the proportions of the placental labyrinth composed of trophoblast, maternal blood spaces, F4 or fetal capillaries (Fig. 4A). IGF-II increased the volume of trophoblast (+29%, $P = 0.015$) and that of maternal blood spaces (+46%, $P = 0.035$) within the placental labyrinth (Fig. 4B). The total surface area of trophoblast functioning in exchange was also increased by IGF-II (+39%, $P = 0.037$, Fig. 4C). There was no effect of IGF treatment on syncytiotrophoblast barrier thickness (vehicle, 4.7 ± 0.2 μm; IGF-I, 4.8 ± 0.2 μm; IGF-II, 4.4 ± 0.2 μm). Maternal IGF-I treatment did not affect any placental labyrinthine structural parameter measured.

Exogenous maternal IGFs increase fetal survival

Maternal IGF treatment did not affect total litter size (Table 2). However, the number of resorptions was reduced by T2 IGF-I (−77%, $P = 0.009$) and IGF-II (−60%, $P = 0.01$), while IGF-II also increased the number of viable fetuses (+25%, $P = 0.034$) near term (Table 2). Maternal IGFs did not alter the ratio of females to males (Table 2).

Exogenous maternal IGFs increase fetal growth with IGF-specific effects on fetal body composition

Maternal IGF-I and IGF-II treatment in early to mid-pregnancy increased fetal weight near term by 17% ($P = 0.002$) and 11% ($P = 0.042$), respectively (Table 3). Both maternal T3 IGF treatments significantly skewed the fetal weight distribution to the right (both $P < 0.0005$; Fig. 5A). The percentage F5 of fetuses heavier than 81 g was 5% in controls, 37% in IGF-I, and 19% in IGF-II-treated animals (Fig. 5A). IGF-I treatment increased fetal crown-to-rump length by 9% ($P = 0.014$), as well as abdominal circumference by 10% ($P = 0.05$). IGF-I increased the fetal weight to placental weight ratio by 29% (vehicle, 14.82 ± 0.86; IGF-I, 19.14 ± 0.73; IGF-II, 16.18 ± 0.65; $P < 0.01$). Fetal weight correlated positively with placental weight across all treatments ($r = 0.27$, $P = 0.026$) and within each of the IGF-I and IGF-II treatment groups ($r = 0.44$, $P = 0.042$ and $r = 0.40$, $P = 0.038$, respectively) but not in vehicle-treated dams alone (Fig. 5B). Overall, fetal weight correlated positively with both the mid-sagittal cross-sectional area and the estimated total volume of the placental labyrinth ($r = 0.58$, $P = 0.009$ and $r = 0.43$, $P = 0.006$, respectively), as well as the volume of trophoblast and fetal capillaries in the placental labyrinth ($r = 0.34$, $P = 0.034$ and $r = 0.62$, $P < 0.001$, respectively).

Maternal IGF-I treatment increased fetal carcass weight (+19%, $P = 0.002$), increased the combined weights of fetal kidneys (+20%, $P = 0.028$), caecum (+24%, $P = 0.027$), total gastrointestinal tract (+13.5%, $P = 0.049$), and the combined fetal fat depots (+16%, $P = 0.028$) (Table 3). Conversely, IGF-I reduced the fractional weights of the fetal spleen (−24%, $P = 0.001$), liver (−12.5%, $P = 0.002$), and brain (−18.5%, $P = 0.004$) (Table 3). Both IGF-I and IGF-II increased the weights of the fetal retroperitoneal fat (+24%, $P = 0.004$; +18%, $P = 0.031$, respectively) and combined fetal muscle mass (+22%, $P = 0.008$; +19%, $P = 0.024$, respectively; Table 3). IGF-I and IGF-II also increased the fetal triceps absolute (+29%, $P = 0.001$; +24%, $P = 0.01$, respectively) and relative weights (both +16%, $P < 0.03$, Table 3). Body composition of male and female fetuses was similar and was similarly affected by maternal IGF treatment (data not shown).

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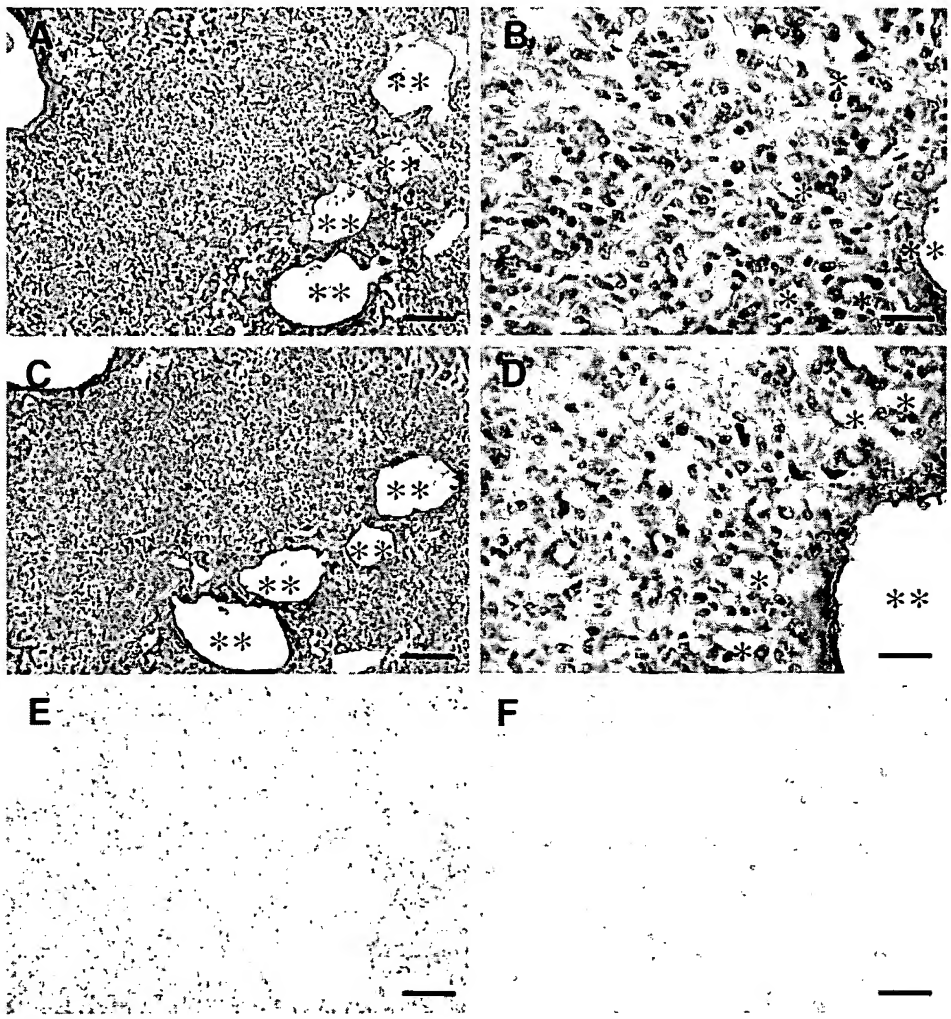

FIG. 2. Representative mid-sagittal serial sections of placentae on d 35 of pregnancy immunolabeled for the type 1 (A and B) and type 2 (C and D) IGF receptors. Representative negative control placental section displayed (E and F). *Two asterisks* indicate maternal blood sinusoids and *single asterisks* indicate maternal blood spaces. *Scale bars*, 400 μm (A, C, and E) and 40 μm (B, D, and F).

Exogenous maternal IGFs increase concentrations of amino acids in the fetal circulation

Maternal IGF-I and IGF-II treatment increased fetal circulating amino acid concentrations (+196%, $P = 0.026$ and +137%, $P = 0.029$, respectively) and maternal IGF-I reduced fetal circulating cholesterol concentrations (−30%, $P = 0.049$) near term (Fig. 6A). There was no effect of treatment on fetal plasma glucose, triglyceride, or free fatty acid concentrations (Fig. 6A).

Exogenous maternal IGF-I, but not IGF-II, alters maternal body composition

Weight gain and body composition analyses were performed to determine whether exogenous IGFs affected the mother. Both exogenous maternal IGF-I and IGF-II did not alter maternal weight gain during or after IGF treatment (Fig. 7), nor total body and lean body mass near term (Table 4). IGF-I reduced maternal interscapular fat depot weight (−25%, $P = 0.028$) and the fractional weights of the perirenal (−32%, $P = 0.05$), retroperitoneal (−33%, $P = 0.037$), and interscapular fat (−28%, $P = 0.01$; Table 4). IGF-I reduced the absolute and fractional weights of the combined adipose depot weights in the mother by approximately 30%, ($P = 0.016$ and $P = 0.007$, respectively). IGF-II did not alter the absolute or relative weights of any maternal organ or tissue examined.

Exogenous maternal IGF treatment does not alter maternal circulating metabolite concentrations

Maternal IGF treatment did not alter circulating concentrations of glucose, free fatty acids, amino acids, triglycerides, or cholesterol in the mother near term (Fig. 6B).

Exogenous maternal IGF treatment and maternal circulating hormone concentrations

To determine whether treatment of the mother during early to mid-pregnancy with IGFs altered maternal circulating estradiol (Fig. 7C) and progesterone (Fig. 7D), their concentrations were determined on d 35 of pregnancy in the additional cohort of guinea pigs in which the plasma IGF and IGFBP concentrations were determined as described above. Treating the mother during early to mid-pregnancy with IGF-I doubled circulating maternal estradiol concentrations in late pregnancy, although this was not quite significant ($P = 0.078$). IGF-I treatment did not alter mid or late pregnancy circulating progesterone concentrations. Exogenous

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TABLE 1. Effect of maternal IGF treatment on placental structure near term

	Vehicle	IGF-I	IGF-II
Placental weight (g)	4.63 ± 0.29^{a,b}	4.11 ± 0.24^{a}	4.84 ± 0.22^{b}
Cross-sectional area labyrinth (mm^2)	98.9 ± 3.8^{a}	112.3 ± 8.9^{a}	126.6 ± 8.3^{b}
Cross-sectional area interlobium (mm^2)	35.6 ± 2.8	32.6 ± 4.3	30.4 ± 2.6
Labyrinth:interlobium	3.10 ± 0.43	3.89 ± 0.44	4.23 ± 0.35
Proportion labyrinth (%)	73.6 ± 1.2^{a}	77.6 ± 1.1^{a,b}	80.5 ± 1.1^{b}
Proportion interlobium (%)	26.4 ± 1.2^{a}	22.4 ± 1.1^{a,b}	19.5 ± 1.1^{b}
Volume labyrinth (cm^3)	3.34 ± 0.25^{a}	3.26 ± 0.23^{a}	4.26 ± 0.23^{b}
Volume interlobium (cm^3)	1.21 ± 0.09	0.95 ± 0.09	1.03 ± 0.08

Data are expressed as mean ± SEM from seven to nine dams per treatment with one to three placentae randomly selected for histological analysis.

Different superscripts denote differences between groups, *a vs. b*, $P < 0.039$.

maternal IGF-II during early to mid-pregnancy increased circulating estradiol concentrations (+150%) in mid-pregnancy and progesterone concentrations in mid (+53%) and late (+83%) pregnancy in the mother; however, these also did not reach statistical significance ($P > 0.08$) (Fig. 7, C and D).

Discussion

The present study demonstrates for the first time that administration of IGF-II to the mother in early to mid-pregnancy increases placental structural and functional capacity by increasing the volume and surface area of the exchange region of the placenta near term, whereas IGF-I has no effect on the placenta. IGF-I, in contrast, reduced maternal adiposity late in pregnancy, whereas IGF-II did not affect maternal body composition. Importantly, however, maternal treatment with either IGF in early to mid-pregnancy substantially reduced fetal resorptions, increased fetal weight, and increased fetal circulating amino acid concentrations near term. Furthermore, administration of IGF-II also increased fetal viability in late pregnancy. This suggests that maternal IGF abundance, particularly that of IGF-II, during the period of early placental growth and development may determine in part the margin of safety between placental capacity to deliver, and fetal demand for, substrates throughout pregnancy.

Specifically, in the current study, administration of 1 mg/kg·d IGFs increased the abundance of maternal circulating IGF-II and IGF-I by 2.5- to 3.4-fold, during early to mid-pregnancy. The concentration of free IGF to IGFBP ratio in the maternal plasma, and hence bioavailable IGF, was also substantially increased. Similar IGF treatment of guinea pigs during early to mid-pregnancy increased placental weight at mid-gestation (41), which was not sustained to near term in the current study. Importantly, however, the functional capacity of the placenta, as indicated by the mid-sagittal cross-sectional area, proportion and volume of the region devoted to exchange (labyrinth) were increased late in gestation, by prior maternal IGF-II treatment. Furthermore, although the composition of this exchange region of the placenta was unaltered by earlier maternal IGF treatment, the total volume of trophoblast and maternal blood spaces, as well as the total surface area of placenta functioning in exchange were increased by IGF-II. As the labyrinth expands at the expense of the interlobium in the second half of pregnancy in the guinea pig (30, 34, 51), together these changes in the structure of the placenta as a result of earlier exogenous maternal IGF-II are suggestive of a more mature placenta and would be expected to increase placental transport capacity. In contrast, maternal exogenous IGF-I had no effect on placental structural development.

Rapid placental structural differentiation and growth occurs in early to mid gestation in all eutherian mammals. In humans and guinea pigs, trophoblasts invade deep within the uterus and its arterioles, extensively remodeling them, to permit increased maternal blood flow to the placenta (32, 52, 53). This ensures delivery of oxygen and nutrients to the placenta, and subsequently to the fetus. The sustained effects of maternal IGF-II supplementation in early to mid-pregnancy on the placenta reported here are the converse of those observed after specific deletion of IGF-II within the placenta. IGF-II is abundantly expressed by invasive trophoblasts of human (54), mouse (55), rat (56), and guinea pig placenta (57). Ablation of placenta-specific *Igf2* gene expression (P0 transcript) in mice reduced the surface area for exchange, increased the exchange barrier thickness and also impaired nutrient transport capacity of the placenta (16, 17).

Reduced maternal circulating IGF-II in mid-pregnancy, as a result of undernutrition in guinea pigs (36), is associated with similar consequences to those of placental *Igf2* gene deletion (17), with a delay and impairment in the functional maturation of the placenta and with reduced fetal growth in both mid and late gestation (37). Together these findings indicate that maternal circulating IGF-II may act in an endocrine fashion to modulate placental development, in addition to any autocrine/paracrine effects of locally produced IGF-II. We suggest that exposure to increased circulating maternal IGF-II in early to mid-pregnancy may provide a foundation of increased placental trophoblast proliferation and invasion of the uterus and its vasculature, which leads to increased volumes of both trophoblast and maternal blood spaces in the placental labyrinth in late gestation. This would be expected to increase maternal blood flow to the placenta and enhance growth of the area devoted to exchange improving placental transfer of oxygen and nutrients to the fetus from the mother. This was consistent with increased circulating fetal amino acid concentrations with earlier maternal IGF treatment, near term. Hence, maternal IGF-II supplementation presumably increased fetal growth and viability predominantly by these actions on the placenta. Current studies in our laboratory are focused on determining whether early maternal IGF treatment increases placental transport of nonmetabolizable analogs of glucose and amino acids in the fetal circulation and tissues and whether treatment affects nutrient partitioning in the mother.

Supplementing the mother during early to mid-pregnancy with either IGF had a sustained positive effect on fetal weight, length, and girth near term, which is consistent with the anabolic effects on the fetus seen at mid-pregnancy after

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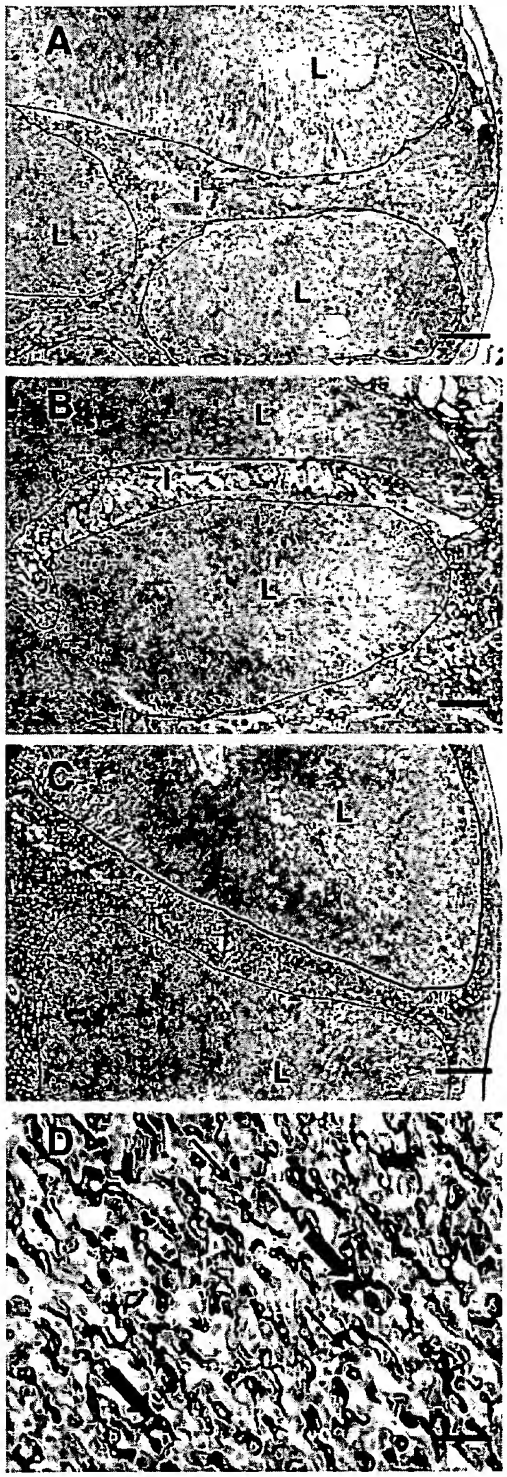

FIG. 3. The effect of exogenous maternal IGF treatment on placental structure. Representative mid-sagittal sections of near-term placentae stained with Masson's Trichrome to distinguish labyrinth and interlobium layers from mothers that had been treated with vehicle (A), IGF-I (B), or IGF-II (C) during early to mid-pregnancy. L, Labyrinth; i, interlobium. *Scale bars*, 400 μm. D, Representative mid-sagittal section of near-term placenta double-labeled and eosin stained to reveal structural components of the placental labyrinth, including fetal trophoblast (*thin arrow*), maternal blood spaces (*asterisks*), and fetal capillaries (*broad arrows*). *Scale bar*, 40 μm.

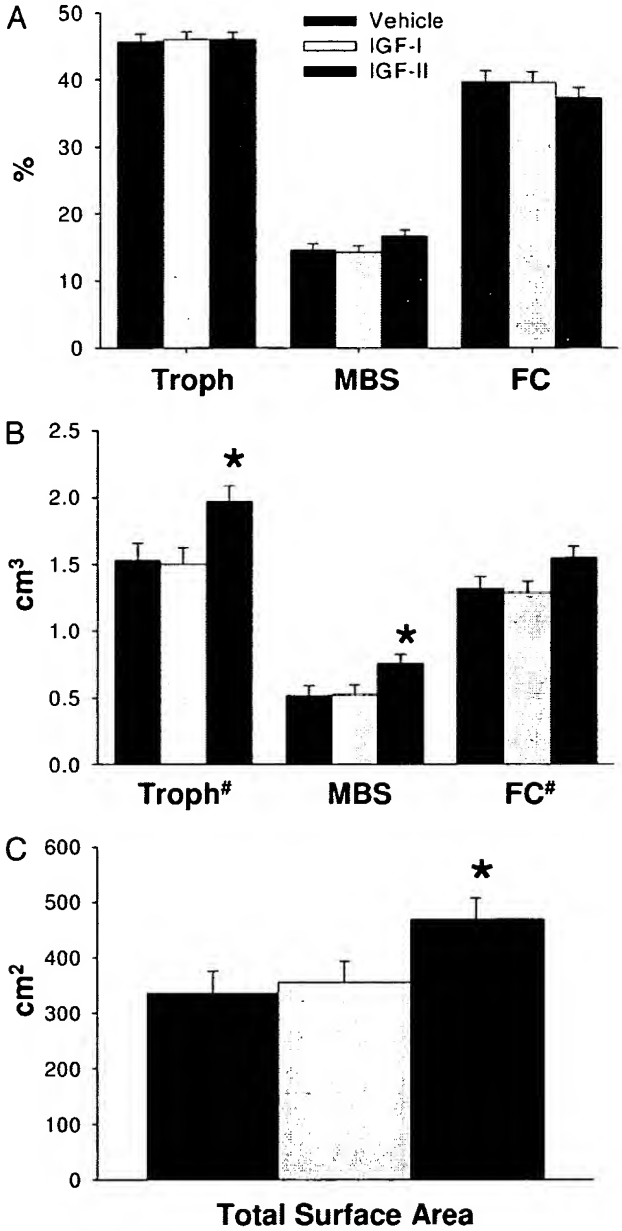

FIG. 4. The effect of exogenous maternal IGFs on structural correlates of placental exchange function near term. Proportions (A) and volumes (B) of fetal trophoblast, maternal blood spaces, and fetal capillaries in the placental labyrinth (exchange region), as well as the total surface area of syncytiotrophoblast for exchange (C). Data are from n = 1–3 placentae from each of seven to nine mothers per treatment. Values are expressed as means ± SEM. *Asterisks* denote a statistically significant difference compared with the vehicle group, $P < 0.05$. #, Positive correlation with fetal weight, $r > 0.34$ and $P < 0.034$.

similar treatment in the guinea pig (41). The increased fetal weight observed with maternal IGF treatment appears to be substantially due to increased muscle mass overall and proportionately for selected muscles and perhaps enhanced fetal bone growth as indicated by increased carcass weights. This may be metabolically beneficial in later life because muscle is an important site for insulin-induced glucose uptake. In-

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TABLE 2. Effect of maternal IGF treatment on litter composition and fetal dimensions near term

	Vehicle	IGF-I	IGF-II
Dams	7	7	9
Fetuses	19	22	30
Females/males	9/10	12/10	14/16
Total litter	3.42 ± 0.1	3.36 ± 0.1	3.67 ± 0.1
Number viable	2.73 ± 0.2^a	3.27 ± 0.9^{a,b}	3.40 ± 0.2^b
Number resorbing	0.68 ± 0.1^a	0.09 ± 0.1^b	0.27 ± 0.1^b

Data are expressed as mean ± SEM.
Different superscripts denote significant differences between groups, $P < 0.05$.

deed, fetal growth restriction in the guinea pig, induced by maternal food restriction and accompanied by reductions in circulating maternal IGF concentrations (36), is characterized by deficits in muscle mass, increased adiposity in the fetus near term (58) and with increased blood pressure and impaired glucose and cholesterol homeostasis in adult offspring (59–61).

TABLE 3. Effect of maternal IGF treatment on fetal weight and body composition near term

	Vehicle	IGF-I	IGF-II
Fetal weight (g)	66.62 ± 2.40^a	77.75 ± 1.96^b	74.03 ± 1.69^b
Crown-rump length (cm)	14.00 ± 0.34^a	15.28 ± 0.28^b	14.77 ± 0.24^{a,b}
Abdominal circumference (cm)	8.82 ± 0.28^a	9.69 ± 0.23^b	9.01 ± 0.20^{a,b}
Head width (cm)	6.81 ± 0.46	7.07 ± 0.39	7.20 ± 0.37
Kidneys (g)	0.59 ± 0.04^a	0.71 ± 0.03^b	0.67 ± 0.03^{a,b}
(% Body weight)	0.89 ± 0.04	0.92 ± 0.03	0.91 ± 0.03
Spleen (g)	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.01
(% Body weight)	0.17 ± 0.01^a	0.13 ± 0.01^b	0.15 ± 0.01^{a,b}
Liver (g)	3.71 ± 0.18	3.77 ± 0.14	3.84 ± 0.13
(% Body weight)	5.6 ± 0.2^a	4.9 ± 0.1^b	5.2 ± 0.1^a
Brain (g)	2.49 ± .07	2.51 ± 0.06	2.52 ± 0.05
(% Body weight)	3.8 ± 0.2^a	3.1 ± 0.1^b	3.5 ± 0.1^{a,b}
Total GI tract (g)	3.33 ± .014^a	3.78 ± 0.11^b	3.59 ± 0.10^{a,b}
(% Body weight)	5.0 ± 0.1	4.9 ± 0.1	4.9 ± 0.1
Caecum (g)	0.37 ± 0.03^a	0.46 ± .02^b	0.40 ± 0.02^{a,b}
(% Body weight)	0.56 ± 0.03	0.59 ± 0.02	0.54 ± 0.02
Total muscle (g)	0.36 ± 0.21^a	0.44 ± 0.16^b	0.43 ± 0.15^b
(% Body weight)	0.56 ± 0.02	0.57 ± 0.02	0.55 ± 0.01
Triceps (g)	0.17 ± 0.01^a	0.22 ± 0.01^b	0.21 ± 0.01^b
(% Body weight)	0.25 ± 0.01^a	0.29 ± 0.008^b	0.29 ± 0.007^a
Total fat (g)	2.39 ± 0.11^a	2.77 ± 0.09^b	2.72 ± 0.08^{a,b}
(% Body weight)	3.6 ± 0.1	3.6 ± 0.1	3.7 ± 0.09
Retroperitoneal fat (g)	0.63 ± 0.04^a	0.78 ± 0.03^b	0.74 ± 0.03^b
(% Body weight)	0.9 ± 0.04	1.0 ± 0.03	1.0 ± 0.03
Carcass (g)	48.68 ± 2.0^a	58.01 ± 1.6^b	53.98 ± 1.5^{a,b}
(% Body weight)	73 ± 0.8	75 ± 0.6	74 ± 0.6

Data expressed as estimated marginal means ± SEM adjusted for the number of viable fetuses per litter. Only tissues that were significantly affected by treatment are shown. *Different superscripts* denote significant difference between groups, *a vs. b*, $P < 0.05$.

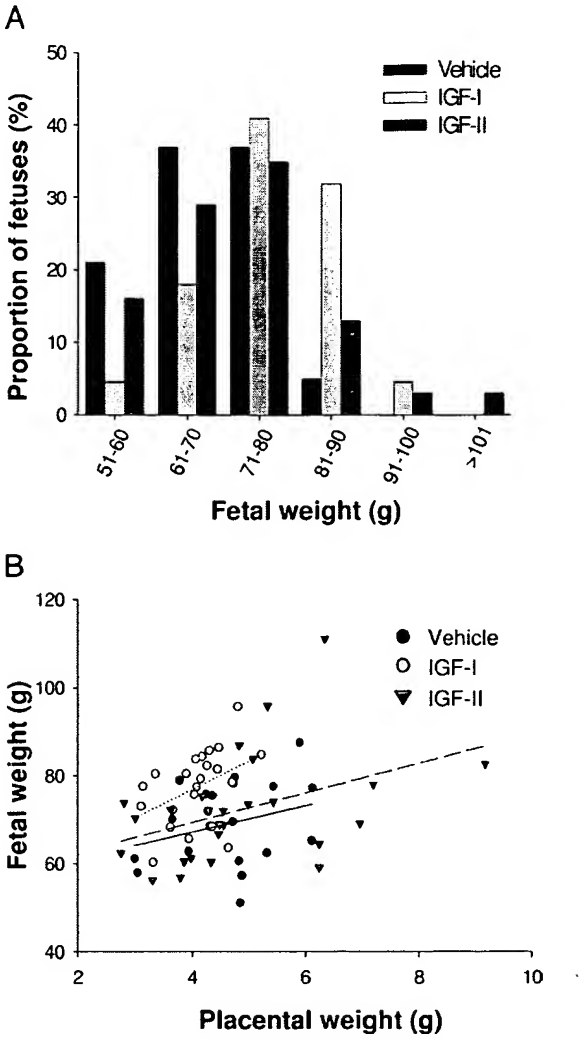

FIG. 5. The effect of exogenous maternal IGF treatment on fetal weight distribution (A) and on the association of fetal weights with placental weights (B). Each fetus from seven to nine mothers per treatment is represented.

The present study suggests that increased maternal IGF-I and IGF-II abundances during early to mid-pregnancy promote fetal growth and viability near term by multiple mechanisms. In addition to direct effects of IGF-II on placental structural development, which in the current study were positively associated with fetal weights, the IGFs may increase nutrient transporter expression (20–22) and/or placental vasodilation (26), which would allow for more substrate to be delivered to the fetus for its growth. The IGFs may also influence placental metabolism and function, which, in turn, may drive major physiological adaptations to pregnancy in the mother, including the development of insulin resistance to divert nutrients to the conceptus (62–64). This has been attributed to placental production of hormones including estrogen, progesterone, and placental lactogen (64, 65) that reduce maternal insulin secretion (64, 66) and antagonize the effects of insulin on maternal tissues, including

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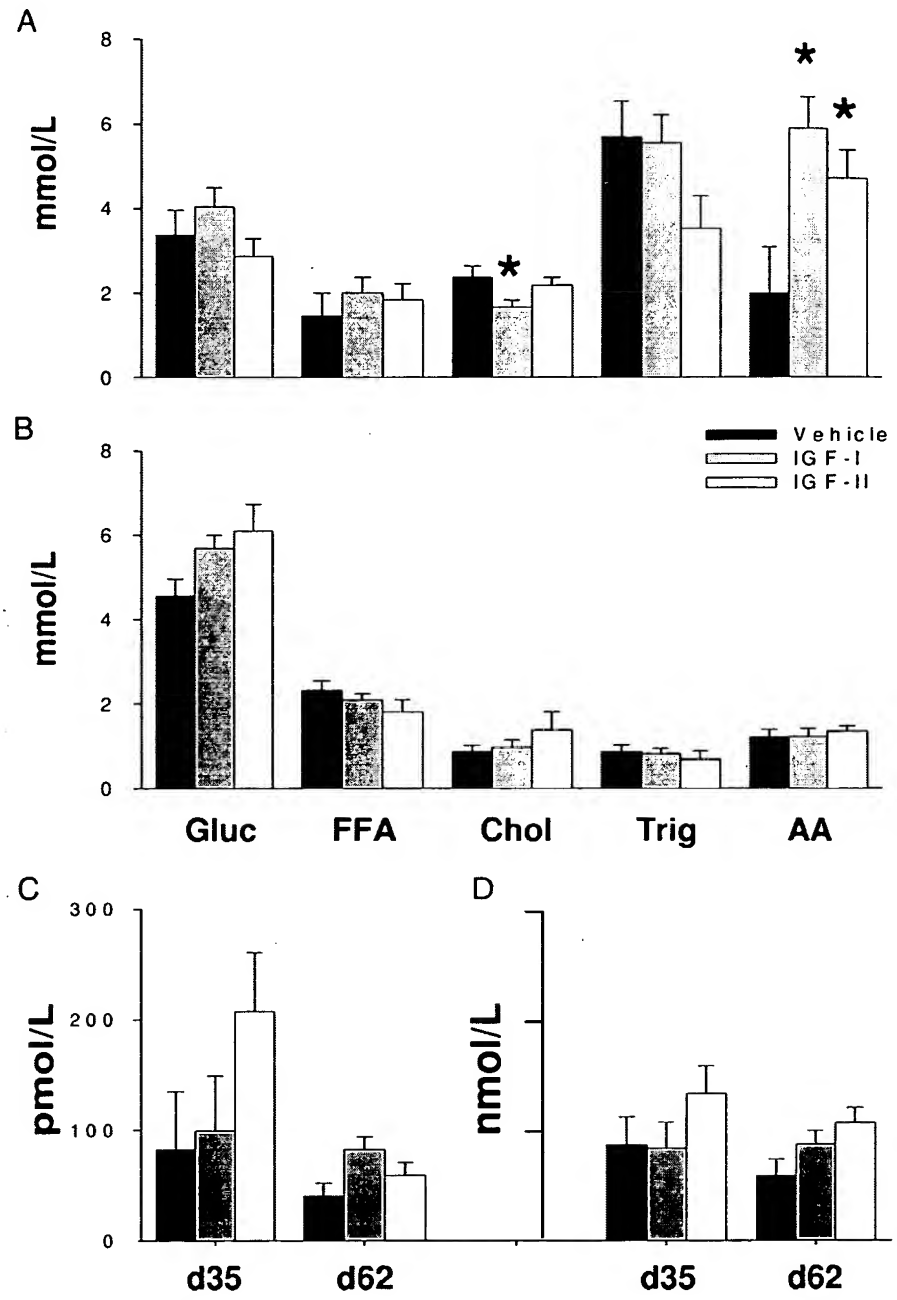

FIG. 6. The effect of exogenous maternal IGFs on circulating metabolites in the fetus (A) and mother (B) near term and estradiol (C) and progesterone (D) in the mother on d 35 and 62 of pregnancy. Fetal data are from all fetuses of six to eight mothers per treatment, and values are expressed as estimated marginal means adjusted for the number of viable pups ± SEM. Maternal data are from six to eight mothers per treatment, and values are expressed as means ± SEM. AA, mino acids; Chol, cholesterol; d35, d 35 of pregnancy; d62, d 62 of pregnancy; FFA, free fatty acids; Gluc, glucose; Trig, triglycerides. *Asterisks* denote a statistically significant difference compared with the vehicle group, $P < 0.049$.

fat deposition (65). Treatment of the mother with IGF-II enhanced placental weight in mid-pregnancy (41) and is accompanied by elevated maternal circulating estradiol and progesterone concentrations, although these were not significant. This would be expected to amplify insulin resistance and other adaptations such as fat deposition in the mother. Consistent with this, exogenous IGF-II during early to mid-pregnancy in guinea pigs increased maternal interscapular adiposity at mid-pregnancy (41) and there was a trend to raised maternal circulating glucose concentrations near term. These increased maternal adipose stores were depleted to normal by late pregnancy in the current study, which may have further enhanced nutrient availability for the fetus, either directly or indirectly. This suggests that IGF-II acts on the placenta to increase fetal growth, by sustainedly promoting placental development, but additionally may enhance maternal physiological adaptation to pregnancy.

The mechanism by which increased maternal IGF-I abundance in early to mid-pregnancy sustainedly promotes fetal growth is less clear. The enhanced placental weight at mid-gestation by prior maternal IGF-I treatment (41), which is no longer apparent in late gestation, may have had persistent effects on the fetus that increased fetal growth near term. In addition, unlike IGF-II, IGF-I did not increase maternal fat deposition in mid-pregnancy (41) and in fact reduced fat depot weights near term. Reduced perirenal fat weight was associated with increased maternal circulating progesterone. Reduced adiposity may reflect increased mobilization

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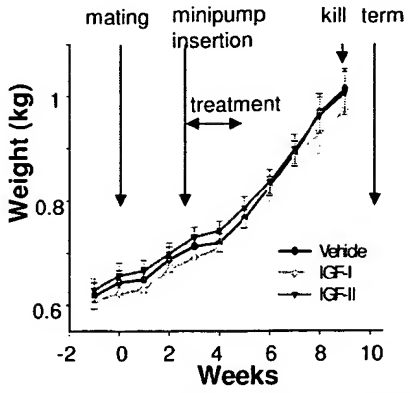

FIG. 7. The effect of exogenous IGFs on maternal weight gain during pregnancy. Female guinea pigs were weighed three times weekly during the study to determine an average weekly weight, from 1 wk before mating and during pregnancy up until kill. Minipumps were inserted on d 20 of pregnancy to deliver vehicle, IGF-I, or IGF-II for 18 d. Term, which is approximately 67–70 d of pregnancy, is denoted on the graph. Data are from seven to nine dams per treatment, and values are expressed as means ± SEM.

and/or reduced deposition during pregnancy, which may have increased substrate availability in the maternal circulation for fetal growth. This has been observed in growth hormone-treated pigs where maternal circulating IGF-I concentration was elevated and associated with reductions in weight of maternal backfat depots (67). Another possible explanation is that larger fetuses of IGF-I-treated dams may signal to the mother via nutrient sensors in the fetal circulation (such as IGFs and insulin), to influence placental metabolism and increase mobilization of maternal adipose tissue stores late in pregnancy.

These differential IGF effects may reflect their distinct interactions with various receptors, because IGF-I binds with high affinity to the IGF1R but negligibly to IGF2R. In contrast, IGF-II binds to both these receptors, as well as to the insulin receptor. In the current study, during mid-pregnancy, the guinea pig placenta ubiquitously expressed both IGF receptor proteins. More importantly, however, at the time of IGF treatment, IGF1R and IGF2R were localized to the apical surface of trophoblasts, within large maternal blood vessels and blood spaces of the labyrinth. In addition, insulin binding sites have preciously been identified in trophoblast of the guinea pig placenta (68–70). This pattern of expression is consistent with the localization of all three receptors to placental trophoblasts in humans and rats (56, 71–77) and abundant expression of IGF1R and IGF2R in invasive trophoblast populations within the human decidua and its vasculature (75).

The specific effects of IGF-II on the placenta, which were not evident in IGF-I-treated animals, suggest that IGF-II actions on the placenta may be mediated by the insulin receptor, which has been implicated in mediating IGF-II effects on fetal growth (78) or by the IGF2R, which it binds with much greater affinity than the IGF1R. There is evidence to suggest that IGF-II acts through IGF2R to promote trophoblast migration and invasion (79, 80), and placental angiogenesis and vascular remodeling (81). IGF-II then, indirectly at least, may enhance placental function by increasing blood supply to the placenta. In contrast, the effects of maternal IGF-I treatment are likely to have been mediated by the IGF1R, particularly because this treatment also reduced IGF-II in the mother.

In conclusion, increased maternal IGF-II in early pregnancy sustainedly promotes placental structural and functional capacity and fetal growth and viability, whereas IGF-I appears to act through the mother to enhance fetal growth to near term. This suggests sustained major and complementary roles in placental and fetal growth for increased circulating IGFs in the mother in early pregnancy.

TABLE 4. Effect of maternal IGF treatment on maternal adipose tissue weights near term

	Vehicle	IGF-I	IGF-II
Number of dams	7	7	9
Weight at d62	978 ± 23	1012 ± 34	971 ± 36
Uterus and contents	242 ± 20	250 ± 44	242 ± 38
Net body mass	736 ± 21	761 ± 62	725 ± 19
Lean body mass	711 ± 19	744 ± 62	702 ± 18
Total fat (g)	25.08 ± 2.3^{a}	17.89 ± 1.6^{b}	23.14 ± 1.0^{a,b}
(% Body weight)	3.4 ± 0.3^{a}	2.4 ± 0.3^{b}	3.2 ± 0.09^{a,b}
Perirenal fat (g)	5.27 ± 0.8	3.50 ± 0.5	4.72 ± 0.4
(% Body weight)	0.71 ± 0.1^{a}	0.48 ± 0.08^{b}	0.66 ± 0.05^{a,b}
Retroperitoneal fat (g)	8.96 ± 0.9^{a}	6.27 ± 0.8^{b}	8.47 ± 0.6^{a,b}
(% Body weight)	1.2 ± 0.1^{a}	0.85 ± 0.1^{b}	1.2 ± 0.06^{a}
Interscapular fat (g)	10.85 ± 0.9^{a}	8.11 ± 0.6^{b}	9.95 ± 0.4^{a,b}
(% Body weight)	1.5 ± 0.1^{a}	1.1 ± 0.08^{b}	1.4 ± 0.06^{a}

Data expressed as means ± SEM. Only tissues that were significantly affected by treatment are shown. Net body mass is weight at postmortem minus the uterus and contents. Lean body mass is net body mass minus total fat. Tissue weight was calculated as a percentage of net body mass. *Different superscripts* denote significant difference between groups, ^{a} *vs.* ^{b}, $P < 0.05$.

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Maternal Food Restriction Reduces the Exchange Surface Area and Increases the Barrier Thickness of the Placenta in the Guinea-pig

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The extent to which maternal nutrition influences fetal growth through effects on placental functional development is unclear. Poor maternal nutrition is a major cause of poor fetal growth which increases neonatal morbidity and mortality, and may also increase the risk of several adult-onset diseases. We have therefore characterized the ontogeny of structural determinants of function in the placenta in guinea-pigs fed ad libitum or food restricted from before and during pregnancy. Guinea-pigs were killed at days 30 and 60 (term=67 days) of pregnancy. In ad libitum fed animals, the surface density (surface area/g placental labyrinth), which is a measure of the convolution of the exchange surface, doubled, while total surface area increased 18-fold between mid and late gestation. Concomitantly, the arithmetic mean barrier thickness to diffusion across trophoblast decreased by 68 per cent. Late in gestation, food restriction reduced the proportion of the placenta devoted to exchange (labyrinth) by 70 per cent ($P<0.04$) and the weight of the placental labyrinth by 45 per cent ($P=0.001$). Maternal food restriction also reduced the total placental surface area for exchange by 36 per cent at day 30 ($P=0.02$) and 60 per cent at day 60 ($P<0.0005$) of gestation, and the surface density of trophoblast by 36 per cent at day 30 ($P=0.01$) and 29 per cent at day 60 ($P=0.005$) of gestation. The arithmetic mean barrier thickness for diffusion was increased by maternal food restriction at both gestational ages (day 30, +37 per cent, $P=0.008$, and day 60, +40 per cent, $P=0.01$). These findings suggest that maternal food restriction not only reduces fetal and placental weights, but also induces structural alterations in the placenta that indicate functional impairment beyond what would be expected for the reduction in its weight.
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INTRODUCTION

Maternal nutrition, which determines the availability of nutrients for transfer to the fetus by the placenta, is a major determinant of fetal growth and outcome. In many developing countries, chronically poor maternal nutrition is a major cause of intrauterine growth restriction (Murthy, Agarwal and Khanna, 1976). In Australia, nearly one-third of women who deliver babies of low birthweight (<2500 g) at term, suffer from eating disorders (Conti, Abraham and Taylor, 1998).

While maternal undernutrition may restrict fetal growth in part by also restricting placental growth and functional development, the extent to which this occurs is unclear. The effects of maternal undernutrition on placental weight are variable depending on the timing and duration of nutritional deprivation. Women subjected to the Dutch Famine of 1944–45 in the third trimester of pregnancy gave birth to babies of low birthweight, with light placentae and an unaltered ratio of placental weight to birthweight compared with unexposed women (Lumey, 1998). However, in women exposed to famine during the first trimester of pregnancy, placental weight at term was increased, with birthweight remaining the same as that in unexposed women (Lumey, 1998). Similarly, undernutrition during early to mid pregnancy in sheep increased the placental to fetal weight ratio by increasing placental weight at term without any effect on fetal weight (Heasman et al., 1998) suggesting that undernutrition during early pregnancy maintains or increases placental weight at term. It is unclear whether this increased relative placental size reflects a compensatory attempt to maintain or increase placental function or the development of a less efficient placenta in response to maternal undernutrition.

Chronic undernutrition is present from before as well as throughout pregnancy in many populations around the world, but its impact on placental development and fetal growth has not been determined. The response of the placenta to maternal

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food restriction at different times during pregnancy is influenced by maternal body weight at conception in sheep, where light ewes at mating bear lighter fetuses and lambs (Kelly, 1992; McCrabb, Egan and Hosking, 1992). Similarly in women, prepregnant weight has been shown to be a more important determinant of placental size than weight gain during pregnancy (Stevens-Simon, Metlay and McAnarney, 1995).

Previously, we examined the effect on fetal and placental growth of restricting nutrition from 4 weeks before pregnancy and during pregnancy in the guinea-pig. This should impair the capacity of the mother to deliver nutrients to the fetus to a greater extent because her own fuel reserves are depleted prior to conception (Sohlstrom et al., 1998). The guinea-pig was used because its placenta is more similar to the human placenta than that of other non-primate species, being haemomonochorial, although it is labyrinthine rather than villous in structure and this species is litter-bearing in contrast to the human. The guinea-pig placenta is comprised of a labyrinth, which contains both fetal capillaries and maternal blood sinuses and provides the means for exchange between the two circulations, and an interlobium. The latter contains only maternal blood sinuses within syncytiotrophoblast and is the site where much of the metabolic activity of the placenta is thought to occur (Kaufmann and Davidoff, 1977). The labyrinth is formed when fetal mesenchymal cells invade into the syncytiotrophoblast, a process which continues throughout gestation at the expense of the interlobium (Kaufmann and Davidoff, 1977).

Maternal food restriction from before and throughout pregnancy in guinea-pigs reduced fetal, and to a lesser extent placental, growth in mid and late gestation, increasing the placental to fetal weight ratio (Sohlstrom et al., 1998). This may indicate either placental compensatory growth to minimize fetal growth restriction or reduced placental function per gram of placenta. Other studies on the effect of undernutrition imposed during pregnancy in guinea-pigs have found that a 40 per cent reduction in food intake from the day of mating resulted in significantly reduced fetal weight, total placental weight, and weight of the placental labyrinth by late pregnancy (Dwyer et al., 1992). However, the ratio of placental weight to fetal weight was increased in mid pregnancy but had recovered to that of ad libitum fed controls by term, suggesting that fetal growth had caught up with placental growth late in gestation.

In this study we wished to determine if maternal undernutrition from 4 weeks before and during pregnancy alters placental structure, as well as reducing placental weight while increasing the ratio of placental weight to fetal weight in the guinea-pig. In particular, the effect of maternal undernutrition on the ontogeny of the thickness of the barrier to diffusion and the surface area of this barrier for exchange and their relationship to fetal size was examined. In this study, barrier thickness was essentially the thickness of syncytiotrophoblast through which all solute exchange between maternal and fetal circulations occurs and, together with syncytiotrophoblast surface area for exchange, is a determinant of placental substrate transfer capacity (Amaladoss and Burton, 1985).

MATERIALS AND METHODS

Animals

Forty virgin guinea-pigs (IMVS Coloured strain) aged 3–4 months were obtained from the Gilles Plains Animal Resource Centre (South Australia) and housed individually in wire bottom cages. They were maintained in a room with a 12 : 12 h light : dark cycle at 25°C. Guinea-pigs were fed a Guinea-pig/Rabbit ration (Milling Industries Stockfeeds, Murray Bridge, SA, Australia) supplemented with Vitamin E (165 mg/kg). Tap water containing Vitamin C (400 mg/l) was available ad libitum.

Female guinea-pigs were divided into two groups of similar mean weight. One group continued to feed ad libitum while the other was fed 70 per cent of the ad libitum intake. All females were weighed three times per week, as was the food in the hopper of the ad libitum fed animals before feeding each day to determine the amount of food consumed per gram body weight. The food restricted group received 70 per cent of the ad libitum intake per gram body weight. After 4 weeks on these diets females which had a ruptured vaginal membrane were deemed to be in oestrus and a male of the same strain was put in the cage overnight and removed next morning. The presence of a vaginal copulatory plug on the tray below the cage was considered as evidence of mating and this was designated day 1 of pregnancy. Food restricted animals continued to be fed 70 per cent of the *ad libitum* intake per gram bodyweight for the first 34 days of pregnancy and from days 35–60 food was increased to 90 per cent of the ad libitum intake.

Female guinea-pigs were killed at either day 30 or 60 of pregnancy (term is 67 days) by an intraperitoneal overdose of sodium pentobarbitone (325 mg/ml, Lethabarb, Virbac, Australia). The uterus was dissected and the fetuses weighed. The sub-placenta was trimmed from the placenta prior to weighing and measuring its thickness and both the long and short diameters. Placental volume was calculated from these placental dimensions. Placentae were immediately dissected into sagittal slices and wedges and immersion fixed in 4 per cent (g/v) paraformaldehyde/0.25 per cent (v/v) glutaraldehyde/2.5 per cent (g/v) polyvinyl pyrrolidone in 70 mmol/l sodium phosphate buffer for 3 days at 4°C. Placentae were then immersed for 2 days in four changes of 10 vol. each of 0.15 mol/l NaCl, 70 mmol/l phosphate buffered saline (PBS pH 7.4) to remove excess aldehydes and processed. Saggital slices were embedded flat on the cut surface while wedges were embedded with a random orientation, after which 7 μm sections were cut.

For subsequent placental structural analyses at least two placentae were randomly selected, without bias as to horn or position therein, from each of four mothers in each nutritional group, at each gestational age, providing two to three replicates from within each of 16 mothers. These animals are a subset of a larger study of the effect of food restriction on changes in the maternal insulin-like growth factor axis during pregnancy (Sohlstrom et al., 1998).

Immunohistochemistry

Randomly oriented sections were double labelled with mouse monoclonal antibodies to human vimentin (3B4, Dako, Denmark) and human pan cytokeratin (C2562, Sigma, St Louis, USA) to identify fetal capillaries and trophoblast, respectively. The immunohistochemistry employed a triple layer technique for each primary antibody, sequentially. Briefly, the sections were first subjected to antigen retrieval by incubating at 37°C for 15 min in 0.03 per cent protease (Sigma). Sections were then blocked for endogenous peroxidase activity with 3 per cent hydrogen peroxide and for non-specific binding of protein with protein blocking serum (Vector, Burlingame, California, USA) for 30 min each. The first primary antibody applied was the mouse anti-vimentin, diluted in PBS with 10 per cent guinea-pig serum and 1 per cent BSA and incubated overnight at room temperature. The sections were washed three times in PBS and the biotinylated goat anti-mouse secondary anti IgG $F(ab')_2$ fragment (Dako, Denmark) was applied for 30 min, following which the sections were again thoroughly washed. The streptavidin–peroxidase label (Zymed, San Francisco, USA) was applied for 30 min and the sections were washed as above. A black precipitate was developed at the site of vimentin binding to the antibodies by incubating with diaminobenzidine (DAB) with 2 per cent ammonium nickel (II) sulphate (Sigma, St Louis, USA). The same process was then used for the second antibody (anti-cytokeratin) but the chromogen step did not include nickel leaving a brown precipitate. Negative controls used irrelevant mouse IgG in place of the primary antibodies or the primary antibody diluent on its own.

Placental morphometry

Sagittal placental sections were stained with haematoxylin and eosin. The proportions of placental labyrinth, interlobium and 'other' tissue, mainly large blood vessels, were quantified using point counting with an isotropic L-36 Merz transparent grid placed on the monitor screen. Sections were examined with a 4 × objective lens, and a 3.3 ocular lens, on an Olympus BH2 microscope equipped with a Video Image Analysis system using Video Pro software (Leading Edge, Australia). Ten fields (360 points) were counted in each section, with the first field location chosen at random and subsequent adjacent sections systematically selected 1 mm apart with the aid of the stage micrometer. The volume densities of placental labyrinth and interlobium were calculated using the following formula:

$$\text{Volume Density, } V_d = P_a / P_T$$

where P_a is the total number of points falling on that component and P_T is the total number of points applied to the section (Weibel, 1979). The weights of placental labyrinth, interlobium and other components were calculated by multiplying placental weight by the volume density of each component.

Immunohistochemically double-labelled sections from randomly oriented blocks were examined with a 20 × objective lens, and a 3.3 ocular lens, on the same imaging system as above. The labyrinthine region of each placenta was morphometrically analysed for the volume densities (proportions) of trophoblasts, fetal capillaries and maternal blood space using point counting as above except that fields were systematically selected 0.5 mm apart. The number of points necessary to achieve a standard error of 10 per cent was calculated from a preliminary study using a nomogram relating test point number and volume density (Weibel, 1979). One section, randomly selected, was counted for each placenta. The volume density of each labyrinthine component was calculated using the same formula as above. Volumes of each labyrinthine component were calculated, assuming that 1 g of placenta occupies 1 cm^3, by multiplying the volume density by weight of placental labyrinth.

The surface density (surface area per gram of placenta) of trophoblast was measured by line intercept counting on the same grid on the same fields and calculated taking into account the total magnification on the monitor using the formula:

$$\text{Surface Density, } S_v = 2 \times I_a / L_T$$

where I_a is the number of intercepts with the line and L_T is the total length of the lines applied (Weibel, 1979). Total surface area was calculated by multiplying the surface density by total placental weight by the proportion of the placenta that was labyrinth.

The arithmetic mean barrier to diffusion was calculated using the formula:

$$\text{Barrier Thickness, } B_T = V_d / S_v$$

where V_d is the volume density of trophoblast and S_v is the surface density of trophoblast (Weibel, 1979). The barrier thickness then, is the thickness of syncytiotrophoblast. The reproducibility of the method was determined by repeating the observations on one section five times. The variation between each was less than 7 per cent.

Statistics

Data from ad libitum fed animals at both gestational ages were analysed by an unbalanced repeated measures, mixed model analysis of variance with structured co-variance matrices (BMDP statistical software, Program 5V, Los Angeles, USA) with gestational age (two levels) and fetus (three levels) as repeated measures factors, and litter size as a co-variate, to identify structural changes from mid to late gestation. Similarly, data from animals in each nutritional group at both gestational ages were analysed as above with maternal nutrition (two levels) as a between factor, gestational age (two levels) and fetus (three levels) as repeated measures factors with litter size as a co-variate. If it was established that litter size had no effect

on particular measurements taken, these analyses were repeated without litter size as a co-variate. Bivariate Pearson two-tailed correlations were performed to relate morphometric parameters with fetal and placental weights and placental size using SPSS (SPSS statistical software, SPSS, Chicago, USA) first in ad libitum fed animals at both gestational ages together, and then data from both nutritional groups from days 30 and 60 of gestation were analysed separately. Differences between groups and correlations were considered statistically significant if $P<0.05$.

This study was approved by the Animal Ethics Committee of Adelaide University and complied with the National Health and Medical Research Council's guidelines on the treatment of animals in research.

RESULTS

Changes in maternal weight and food consumption throughout the study

Maternal weights were similar for both ad libitum fed and food restricted guinea-pigs at entry into the study (Sohlstrom et al., 1998) [Figure 1(A)]. Food restriction reduced the gain in maternal weight seen in ad libitum fed guinea-pigs both before and throughout pregnancy ($P<0.0005$), with the greatest effect late in pregnancy when fetal growth was most rapid [Figure 1(A)]. Food consumption rose slowly during the study as maternal weight increased [Figure 1(B)]. The actual food consumption of food restricted dams at day 1 of pregnancy was 62.5 per cent of that consumed by ad libitum fed animals. Food restricted animals continued to be fed at 70 per cent of ad libitum fed animals per gram bodyweight up to day 34 of pregnancy which corresponded to 60 per cent of ad libitum intake and from then on food consumption was increased to 90 per cent of ad libitum intake per gram bodyweight which corresponded to 64 per cent of ad libitum intake. Food restricted dams were 17 per cent lighter ($P=0.043$) at day 30 of pregnancy and 28 per cent lighter ($P<0.0005$) at day 60 of pregnancy than ad libitum fed dams. Food restriction reduced carcass net weight (bodyweight minus uterus and its contents) by 15 per cent ($P=0.051$) at day 30 of pregnancy and by 23 per cent ($P=0.001$) at day 60 of pregnancy.

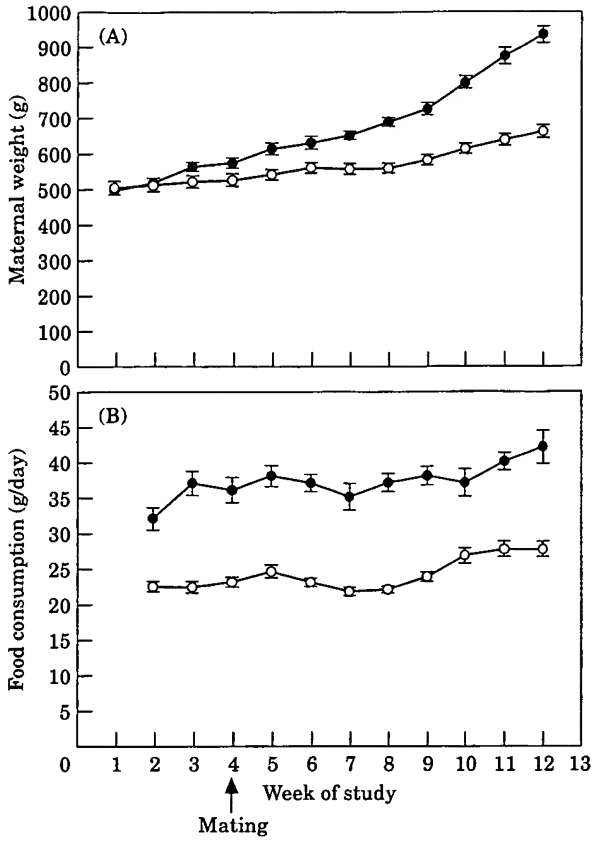

Figure 1. Maternal weight and food consumption throughout the study. (A) Maternal weight in ad libitum fed (●) and food restricted (○) guinea-pigs from 4 weeks before and throughout pregnancy. Effect of pregnancy ($P<0.0005$), maternal food restriction ($P<0.0005$) and the interaction of pregnancy with maternal food restriction ($P<0.0005$) were indicated by repeated measures ANOVA. (B) Food consumption (g/day) in ad libitum fed (●) and food restricted guinea-pigs (○) from before and during pregnancy.

Ontogenic changes in placenta from mid to late pregnancy in ad libitum fed guinea-pigs

Between mid and late gestation in ad libitum fed guinea-pigs, fetal weight increased 49-fold, while placental weight increased six-fold, (both $P=0.00005$) (Table 1). The ratio of placental to fetal weight declined by 87 per cent ($P=0.00005$) during the latter half of gestation (Table 1). Average placental diameter doubled, placental depth increased by over twofold and placental volume increased more than eight-fold between mid and late gestation (all $P=0.00005$) (Table 1).

Between mid and late gestation, the proportion of the placenta that was occupied by the labyrinth increased by 32 per cent ($P=0.0012$) while that occupied by interlobium decreased by 42 per cent ($P=0.001$) (Table 2). The weight of the placental labyrinth increased nearly fivefold while that of interlobium increased nearly four-fold (both $P=0.00005$) between these stages of gestation (Table 2).

The placental labyrinth underwent significant structural change from mid to late gestation. The volume density of trophoblast declined by 31 per cent ($P=0.00005$) while the volume of trophoblast within it increased eight-fold ($P=0.00005$) (Table 3). The volume density of the maternal blood space nearly doubled ($P=0.0408$) while its volume increased 12-fold ($P=0.00005$) in the latter half of gestation (Table 3). The volume density of the fetal capillaries in the labyrinth increased by 36 per cent ($P<0.005$) while their volume increased 18-fold ($P=0.00005$) during this time (Table 3).

Table 1. Effect of nutrition on guinea-pigs at days 30 and 60 of pregnancy

	Ad libitum fed day 30 $n=4$[a]	Food restricted day 30 $n=4$[b]	Ad libitum fed day 60 $n=4$[b]	Food restricted day 60 $n=4$[c]
Fetal weight	1.43 ± 0.17	1.01 ± 0.06**	69.9 ± 1.8	45.2 ± 6.3††
Placental weight	0.73 ± 0.05	0.59 ± 0.04**	4.81 ± 0.35	3.33 ± 0.19††
Ratio of placental to fetal weight/dam	0.52 ± 0.04	0.59 ± 0.02	0.07 ± 0.0004	0.08 ± 0.0008
Litter size	3.25 ± 0.29	2.75 ± 0.29*	2.75 ± 0.29	2.25 ± 0.29†
Placental diameter (mm)	14.31 ± 0.53	14.33 ± 0.47	27.53 ± 1.0	24.56 ± 0.8††
Placental depth (mm)	2.61 ± 0.41	1.95 ± 0.06	6.03 ± 0.32	5.1 ± 0.58
Placental volume (cm^2)	0.42 ± 0.05	0.32 ± 0.03*	3.52 ± 0.15	2.44 ± 0.33††

[a] $n=4$ dams with 2–4 fetuses and placentae within each to a total of 13; [b] $n=4$ dams with 2–3 fetuses and placentae within each to a total of 11; [c] $n=4$ dams with 2–3 fetuses and placentae within each to a total of 9.
All data are means ± SEM. Data for each gestational age were analysed by repeated measures ANOVA.
*Denotes significant difference between nutritional groups at day 30 of pregnancy, $P<0.05$.
**Denotes significant difference between nutritional groups at day 30 of pregnancy, $P<0.01$.
†Denotes significant difference between nutritional groups at day 60 of pregnancy, $P<0.05$.
††Denotes significant difference between nutritional groups at day 60 of pregnancy, $P<0.01$.

Table 2. Ontogeny of, and effect of nutrition on, the proportion of the placenta occupied by labyrinth and interlobium at days 30 and 60 of pregnancy

	Ad libitum fed day 30 $n=4$[a]	Food restricted day 30 $n=4$[b]	Ad libitum fed day 60 $n=4$[a]	Food restricted day 60 $n=4$[b]
Percentage of placenta as labyrinth	56.0 ± 4.7	56.3 ± 2.4	73.7 ± 2.4	58.7 ± 4.5††
Weight of labyrinth (g)	0.42 ± 0.05	0.35 ± 0.02	3.45 ± 0.29	1.96 ± 0.25††
Percentage of placenta as interlobium	40.4 ± 4.4	42.2 ± 4.6	23.4 ± 2.1	35.7 ± 4.3††
Weight of interlobium (g)	0.30 ± 0.02	0.23 ± 0.02*	1.10 ± 0.16	1.17 ± 0.11

[a] $n=4$ dams with 2–3 fetuses and placentae within each to a total of 9; [b] $n=4$ dams with 2–3 fetuses and placentae within each to a total of 8.
All data are means ± SEM. Data for each gestational age were analysed by repeated measures ANOVA.
*Denotes significant difference between nutritional groups at day 30 of pregnancy, $P<0.05$.
††Denotes significant difference between nutritional groups at day 60 of pregnancy, $P<0.01$.

The surface density of trophoblast, which is a measure of surface area per gram placental labyrinth, doubled ($P=0.00005$) while total surface area of trophoblast for exchange increased 18-fold ($P=0.00005$) between mid and late gestation (Table 3). The arithmetic mean barrier thickness to diffusion decreased by 68 per cent ($P=0.00005$) in the latter half of gestation (Table 3).

The effect of maternal food restriction on fetal and placental weights

Maternal food restriction reduced fetal weight by 30 per cent ($P<0.008$) and 35 per cent ($P<0.00005$) at 30 and 60 days of gestation, respectively, compared to ad libitum fed controls (Table 1). Undernutrition also reduced placental weight but to a lesser extent (day 30, −20 per cent, $P<0.01$; day 60, −31 per cent, $P<0.00005$) and consequently the ratios of placental weight to fetal weight increased by 13 per cent and 14 per cent at gestational days 30 and 60, respectively (Table 1), although these were not significant. Maternal food restriction did not alter placental diameter or depth at day 30 of gestation, but reduced placental volume by 24 per cent ($P=0.04$). Food restriction reduced placental diameter by 11 per cent ($P=0.009$), depth by 15 per cent ($P=0.09$) and volume by 31 per cent ($P=0.0003$) at day 60 of gestation (Table 1). Litter size was 15 per cent lower ($P<0.05$) at day 30 and 18 per cent lower ($P<0.03$) at day 60 of gestation in food restricted animals (Table 1).

At day 30 fetal weight was positively correlated with litter size ($r=0.703$, $P=0.002$). The ad libitum fed animals had both larger litters and larger fetuses. Fetal weight at day 30 was positively correlated with both placental weight ($r=0.708$, $P=0.001$) and placental volume ($r=0.670$, $P=0.003$). At day 60 of gestation, fetal weight was strongly positively correlated with placental weight ($r=0.875$, $P<0.0005$), placental volume ($r=0.855$, $P<0.0005$), placental diameter ($r=0.719$, $P=0.001$) and, to a lesser extent, placental depth ($r=0.596$, $P=0.01$).

Table 3. Ontogeny of, and effect of maternal nutrition on, labyrinthine placental structure at days 30 and 60 of pregnancy

	Ad libitum fed day 30 $n=4^a$	Food restricted day 30 $n=4^b$	Ad libitum fed day 60 $n=4^a$	Food restricted day 60 $n=4^b$
Volume density of trophoblast	0.58 ± 0.03	0.60 ± 0.01	0.40 ± 0.03	0.25 ± 0.08†
Volume of trophoblast (g)	0.24 ± 0.03	0.21 ± 0.02	1.91 ± 0.27	0.85 ± 0.30††
Volume density of maternal blood space	0.17 ± 0.01	0.12 ± 0.01**	0.26 ± 0.03	0.19 ± 0.02
Volume of maternal blood space (g)	0.09 ± 0.03	0.04 ± 0.01*	1.05 ± 0.18	0.75 ± 0.11†
Volume density of fetal capillaries	0.28 ± 0.05	0.27 ± 0.02	0.38 ± 0.05	0.35 ± 0.02
Volume of fetal capillaries (g)	0.10 ± 0.01	0.10 ± 0.01	1.75 ± 0.10	1.29 ± 0.09†
Surface density/(cm^2/g)	531.3 ± 34.4	390.8 ± 63.3*	1131.6 ± 41.8	805.8 ± 110.3††
Total surface area (cm^2)	222.9 ± 32.6	143.7 ± 28.4*	3928.8 ± 450.9	1568.4 ± 232.6††
Arithmetic mean barrier thickness (μm)	10.9 ± 0.7	17.2 ± 3.3*	3.5 ± 0.1	6.0 ± 1.0†

[a] $n=4$ dams with 2–3 fetuses and placentae within each to a total of 9; [b] $n=4$ dams with 2–3 fetuses and placentae within each to a total of 8.
All data are means ± SEM. Data for each gestational age were analysed by repeated measures ANOVA.
*Denotes significant difference between nutritional groups at day 30 of pregnancy, $P<0.05$.
**Denotes significant difference between nutritional groups at day 30 of pregnancy, $P<0.01$.
†Denotes significant difference between nutritional groups at day 60 of pregnancy, $P<0.05$.
††Denotes significant difference between nutritional groups at day 60 of pregnancy, $P<0.01$.

That is, fetal weight was reduced as each dimension of the placenta was reduced.

The effect of maternal food restriction on proportion and weight of the placenta devoted to exchange

Food restriction did not change the proportion of the placenta which was labyrinthine at mid gestation, but by day 60 this proportion had failed to increase resulting in a 70 per cent reduction ($P<0.04$) compared to ad libitum fed animals (Table 2). The weight of labyrinth in the placenta was lower in food restricted animals at both days 30 and 60 of gestation as a consequence of the significant reduction in placental weight with food restriction, although this was significant only in late gestation (− 43 per cent, $P=0.00005$) (Table 2). Food restriction did not alter the proportion of the placenta that was occupied by interlobium at day 30 of gestation but as a consequence of the lower placental weight with undernutrition the weight of interlobium was decreased by 23 per cent ($P<0.05$) at this time (Table 2). By day 60 of gestation in food restricted animals the proportion of the placenta occupied by interlobium was 53 per cent higher ($P=0.007$) than in ad libitum fed animals (Table 2). Consequently, despite the reduction in placental weight in food restricted animals late in pregnancy the weight of interlobium was unaltered (Table 2).

The proportion and weight of the placental labyrinth were not correlated with fetal weight in mid pregnancy when both nutritional groups were combined. However, by late gestation, fetal weight was positively correlated with the proportion ($r=0.540$, $P<0.03$) and the weight ($r=0.813$, $P<0.0005$) of the placenta that was labyrinth.

The effect of maternal food restriction on labyrinthine placental morphometry

The volume density and weight of trophoblast was unaltered by food restriction at day 30 of gestation. The volume density of trophoblast was 37 per cent lower ($P=0.03$) in placentae from food restricted compared to ad libitum fed animals at day 60 of gestation while the weight of labyrinthine trophoblast was 55 per cent lower ($P=0.01$) (Table 3). Undernutrition reduced the volume density of the maternal blood space by 29 per cent at day 30 of gestation ($P=0.003$) with an accompanying 55 per cent reduction in its weight ($P<0.03$). The weight, but not the volume density, of the maternal blood space was significantly reduced by food restriction late in gestation (− 29 per cent, $P=0.03$) (Table 3). The volume density of fetal capillaries in the placenta was unaffected by restricted maternal nutrition at both gestational ages, but their volume was reduced by 26 per cent ($P<0.03$) in food restricted compared to ad libitum fed animals late in gestation (Table 3).

The surface density of trophoblast, which reflects the exchange surface area per gram of placenta, was reduced by 26 per cent at day 30 ($P<0.03$) and 29 per cent at day 60 ($P=0.01$) of gestation in food restricted animals. The total exchange surface area was reduced by 36 per cent ($P<0.03$) as a consequence of the reduction in surface density in mid gestation. At day 60 of gestation total surface area was reduced by 60 per cent ($P=0.00005$) as a consequence of both the reduction in surface density and the proportion of the placenta occupied by labyrinth, as well as the reduced labyrinthine weight at this time in food restricted animals (Tables 1 and 3). The arithmetic mean barrier thickness for diffusion in the labyrinthine placenta was increased in the undernourished group at both days 30 (+58 per cent, $P<0.03$) and 60 of gestation (+71 per cent) ($P=0.03$) (Table 3).

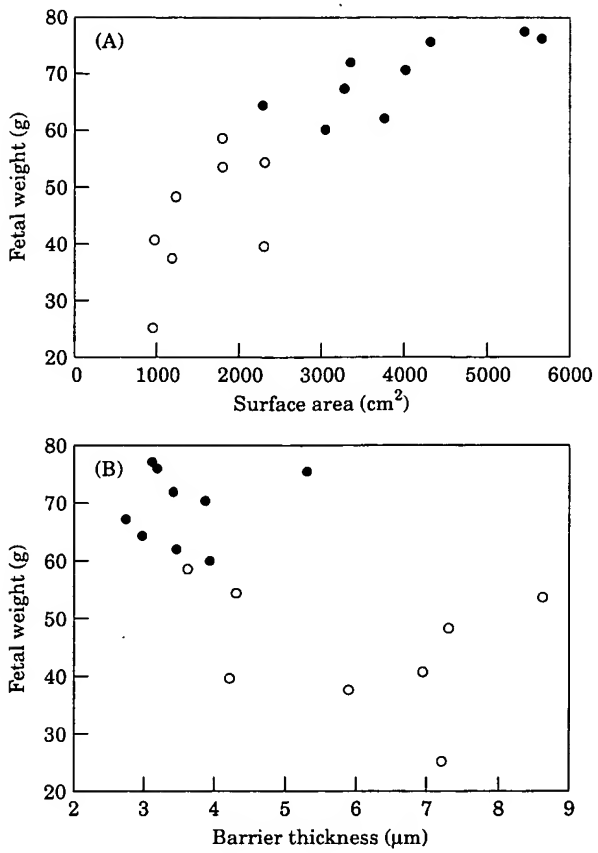

Figure 2. Fetal weight and placental structure in late gestation. Fetal weight and placental structural parameters at day 60 of gestation are shown for offspring of ad libitum fed (●) and food restricted (○) animals. (A) Fetal weight was positively correlated with total apical surface area of syncytiotrophoblast in ad libitum fed animals alone (r=0.776, P=0.014), and in all animals combined (r=0.861, P<0.0005). (B) Fetal weight was negatively correlated with arithmetic mean barrier thickness in all animals combined (r= −0.649, P<0.0005).

In mid-gestation, fetal weight was positively correlated with total surface area (r=0.712, P=0.001) and weight of the maternal blood space (r=0.564, P<0.02) and was negatively correlated with barrier thickness (r= − 0.640, P=0.006) in all offspring from ad libitum and food restricted mothers combined. In late gestation, fetal weight was positively correlated with weight of trophoblast in the labyrinth (r=0.851, P<0.0005), volume of the maternal blood space (r=0.696, P=0.002), weight of fetal capillaries (r=0.607, P=0.01), surface density (r=0.744, P=0.001) and total surface area for exchange (r=0.861, P<0.0005) [Figure 2(A)] in all offspring from ad libitum and food restricted mothers combined. In late gestation fetal weight was negatively correlated with the arithmetic mean barrier thickness (r= − 0.649, P=0.005) [Figure 2(B)]. When offspring from ad libitum fed or food restricted mothers were analysed separately, fetal weight was negatively correlated with total surface area for exchange in offspring of ad libitum fed animals (r=0.776, P=0.014) but not in those from food restricted animals [Figure 2(A)].

DISCUSSION

This study has described, for the first time, the structural changes that occur in the guinea-pig placenta from mid to late gestation, particularly those that relate to placental function. The surface density (surface area of the apical syncytiotrophoblast membrane in contact with maternal blood per gram placental labyrinth) and the total surface area of syncytiotrophoblast, increased between mid and late gestation, which is indicative of increased convolution in the labyrinth, and hence trophoblast differentiation. This is homologous with the tremendous growth, arborization and thinning of terminal villi in the human placenta which occurs also in the latter half of gestation (Jackson, Mayhew and Boyd, 1992). The thickness of the barrier to diffusion, which here is essentially the thickness of syncytiotrophoblast, concomitantly decreased, as occurs in placentae from a wide range of species, including humans, whether the placenta is haemomonochorial or not (Enders, 1982; Jackson, Mayhew and Boyd, 1992). These events, which together have been described as terminal differentiation of trophoblast (Fox, 1997), should increase placental capacity to transfer substrates and hence sustain an increasingly larger fetus and greater fetal growth rate late in gestation. Consistent with this, fetal weight was positively correlated with surface density and surface area of syncytiotrophoblast, and negatively correlated with mean barrier thickness, particularly late in gestation when the increase in fetal weight is most rapid.

Previously, the guinea-pig placenta was described as completing its morphological differentiation by mid gestation (day 36), with subsequent increases in weight and the relative proportion accounted for by the labyrinth for the remainder of gestation (Kaufmann and Davidoff, 1977; Dwyer et al., 1992). Our study agrees with and extends these findings by showing that major determinants of placental function are altered, such that the surface area of syncytiotrophoblast increases, whilst its thickness or part of the barrier to diffusion decreases, during late gestation. We have also substantiated the observation of Dwyer et al. (1992), that maternal food restriction reduces the proportion of the placenta consisting of labyrinth, and hence that devoted to exchange, and suggests retardation of the structural maturation of the placenta. Kaufmann and Davidoff (1977) showed that as the guinea-pig placenta develops, the interlobium is the source of new labyrinth as fetal capillaries invade it. Therefore, maternal food restriction appears to impair the normal conversion of interlobium to labyrinth.

This study has also shown that maternal food restriction from before and during pregnancy alters placental and labyrinthine structure, as well as size, in mid and late gestation, such that its ability to transfer substrates to the fetus may be impaired. The volume density and weight of trophoblast in the placental labyrinth was reduced late in gestation in food restricted guinea-pigs, suggesting a disproportionate reduction in trophoblast proliferation. Placental diameter was also reduced late in gestation in food restricted guinea-pigs, indicating decreased circumferential expansion of the placenta, which has been suggested is a function of the extent of

trophoblast invasion into the decidua (Boyd and Hamilton, 1970). These findings suggest that maternal food restriction may impair trophoblast proliferation and/or invasion, as occurs in pre-eclampsia, where fewer maternal vessels are recruited and placental growth is reduced with concomitant restriction of fetal growth (Khong et al., 1986).

Interestingly, the volume density of fetal capillaries in the placental labyrinth was maintained in food restricted animals, whilst those of maternal blood space and of trophoblast were reduced. This suggests that placentae in food restricted mothers were relatively better vascularized by fetal capillaries. Similar changes occur in human pregnancies complicated by iron-deficiency anaemia where volume of trophoblast and stromal tissue in the villi are reduced, but that of fetal capillaries is unchanged, making them relatively better vascularized (Reshetnikova, Burton and Teleshova, 1995). In the guinea-pig, maternal exposure to hypoxia also increased the volume density of the fetal capillaries of the placenta due to increased capillary branching (Scheffen et al., 1990). Studies on the effect of hypoxia from day 15–64 of pregnancy in guinea-pigs have used both ad libitum fed and pair fed normoxic control groups, since hypoxia reduces food intake (Gilbert et al., 1979; Bacon et al., 1984). In the pair fed group, fetal and placental weights and a range of placental structural characteristics, including mean diffusion distance, were unchanged at day 64 of gestation, but food intake was not actually described (Bacon et al., 1984). In anaemic women there was also a reduction in the harmonic mean barrier thickness of the villous membrane in the placenta (Reshetnikova, Burton and Teleshova, 1995), which is the converse of the increase observed in response to maternal food restriction in the guinea-pig. Therefore, restricted nutrient availability shows some, but not all, of the features elicited by maternal hypoxia.

Maternal food restriction reduced the total surface area for substrate exchange, which correlated with fetal weight overall, suggesting that a reduction in placental capacity to deliver substrates has contributed to the restriction of fetal growth. The thickness of the barrier to diffusion was also substantially increased by maternal food restriction in our study and barrier thickness was negatively correlated with fetal weight overall, consistent with impaired placental substrate transport capacity, possibly due in part, to reduced diffusion through trophoblast cytoplasm. Therefore, maternal food restriction may impair substrate delivery to the fetus, and hence fetal growth, by impairing placental substrate transfer capacity as well as reducing substrate abundance in maternal blood. This is supported by our preliminary evidence that fetal accumulation of ^{3}H-methyl glucose and α-1-^{14}C-aminoisobutyric acid from the maternal circulation is significantly reduced in food restricted guinea-pigs (Sohlstrom et al., 1999).

The effect of undernutrition on placental structure in humans has been little examined. In India, women from four different socioeconomic groups, had variable dietary caloric and protein intakes, which correlated with birth and placental weights, placental volume and surface area, although it is not evident how the latter two parameters were determined (Murthy, Agarwal and Khanna, 1976). The alterations in placental structure induced by maternal food restriction in the guinea-pig appear similar to those seen in growth restricted or pre-eclamptic human pregnancies. In the latter, placental volume, chorionic villus surface area, fetal capillary surface area and the volume density of trophoblast in the placenta are reduced and correlate with birthweight and gestational age (Aherne and Dunnill, 1966; Boyd and Scott, 1985; Teasdale and Jean-Jacques, 1988). Interestingly, guinea-pigs can develop a pre-eclamptic like syndrome when given insufficient nutrition late in pregnancy, especially when accompanied by obesity, which is also a risk factor in humans (Ganaway and Allen, 1971). This syndrome in guinea-pigs has similar laboratory findings to severe pre-eclampsia in women with preceding anorexia, proteinuria, ketosis, acidosis and fatty degeneration of the liver (Ganaway and Allen, 1971; Seidl et al., 1979). We have observed maternal deaths late in pregnancy in the guinea-pig after 2–3 days of anorexia, although we have not examined placental structure or performed urinalyses.

In summary, placentae from food restricted guinea-pigs were smaller and had altered placental structure compared with those from ad libitum fed mothers. These changes, importantly, included an increased arithmetic mean barrier thickness and a decreased surface area of syncytiotrophoblast for exchange. The latter was due to reductions in both placental weight and surface density of syncytiotrophoblast. This implies that not only were these placentae small, but they had a significantly reduced capacity to deliver substrates per gram of placenta as supported by preliminary functional studies (Sohlstrom et al., 1999). We suggest that this is partly due to a retardation of placental maturation, which is present by mid gestation and exacerbated late in gestation. Restriction of nutrition prior to and throughout pregnancy therefore alters placental structure, such that placental capacity for substrate transfer is impaired, suggesting that the resultant fetal growth restriction may be due to placental restriction, as well as reduced maternal substrate availability.

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